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## Dexamethasone regulates gene expression induced by the mechanosensitive P2X7 receptor in osteoblasts

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Graduate Program in Orthodontics  
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Clinical Science  
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DEXAMETHASONE REGULATES GENE EXPRESSION INDUCED BY THE  
MECHANOSENSITIVE P2X7 RECEPTOR IN OSTEOBLASTS

(Thesis format: Monograph)

by

Meena Na

Graduate Orthodontics

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Masters in Clinical Dentistry

The School of Graduate and Postdoctoral Studies  
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London, Ontario, Canada

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## ABSTRACT

Orthodontic tooth movement involves activation of osteoblasts and osteoclasts in response to mechanical forces. Extracellular nucleotides, released in response to mechanical stimuli, signal through P2 receptors in osteoblasts. P2X7 receptors are ATP-gated cation channels implicated in mechanotransduction in bone. Corticosteroids such as dexamethasone exhibit anti-inflammatory effects and their chronic use has been linked to serious side-effects such as osteoporosis. We investigated gene expression following P2X7 receptor activation with benzoylbenzoyl-ATP (P2X7 receptor agonist) in osteoblast precursor cells in the presence or absence of dexamethasone. We hypothesized that dexamethasone inhibits the ability of ATP to stimulate the expression of anabolic (bone forming) genes in osteoblasts. We showed that benzoylbenzoyl-ATP stimulation of *Ptgs2* and *Dmpl* expression is indeed inhibited by dexamethasone. Thus, intracellular events following activation of P2X7 may be important in regulating osteoblast differentiation, maturation and eventual bone formation during orthodontic tooth movement and dexamethasone may adversely affect these processes.

## KEYWORDS

Bone remodeling, orthodontic tooth movement, mechanotransduction, adenosine 5'-triphosphate (ATP), cyclooxygenase-2 (COX-2), knockout mice, nuclear factor of activated T cells (NFAT), osteoblasts, purinergic P2 receptors, purinergic signaling, purinoceptor, P2X, P2X7, *P2rx7*, real-time reverse transcription-polymerase chain reaction (RT-PCR), transcription factors

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## LIST OF ABBREVIATIONS

$\alpha$ MEM	$\alpha$ -minimum essential medium
ADP	adenosine 5'-diphosphate
ALP	alkaline phosphatase
AMP	adenosine 5'-monophosphate
ANOVA	analysis of variance
AP-1	activator protein-1
ATF-4	activating transcription factor 4
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
BSP	bone sialoprotein
BzATP	2',3'-O-(4-benzoylbenzoyl)ATP
cAMP	cyclic adenosine monophosphate
COL1	collagen type 1
COX1	cyclooxygenase 1
COX2	cyclooxygenase 2
FBS	heat-inactivated fetal bovine serum
FGF	fibroblast growth factor
IGF	insulin-like growth factor
IL	interleukin

LPA	lysophosphatidic acid
LPAR	lysophosphatidic acid receptor
M-CSF	macrophage colony stimulating factor
MAPK	mitogen-activated protein kinase
NF- $\kappa$ B	nuclear factor-kappa B
NFATc1-4	nuclear factor of activated T cells, cytoplasmic 1-4
OCN	osteocalcin
OPG	osteoprotegerin
OPN	osteopontin
OSX	osterix
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PGE <sub>2</sub>	prostaglandin E <sub>2</sub>
PKA	protein kinase A
PLA <sub>2</sub>	phospholipase A <sub>2</sub>
PLC	phospholipase C
PTH	parathyroid hormone
RANK	receptor activator of nuclear factor kappa B
RT-PCR	reverse transcriptase polymerase chain reaction
RUNX2	runt-related transcription factor 2

SEM	standard error of the mean
SOST	sclerostin
SOX9	sex-determining region Y (SRY)-box 9
TGF	transforming growth factor
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate

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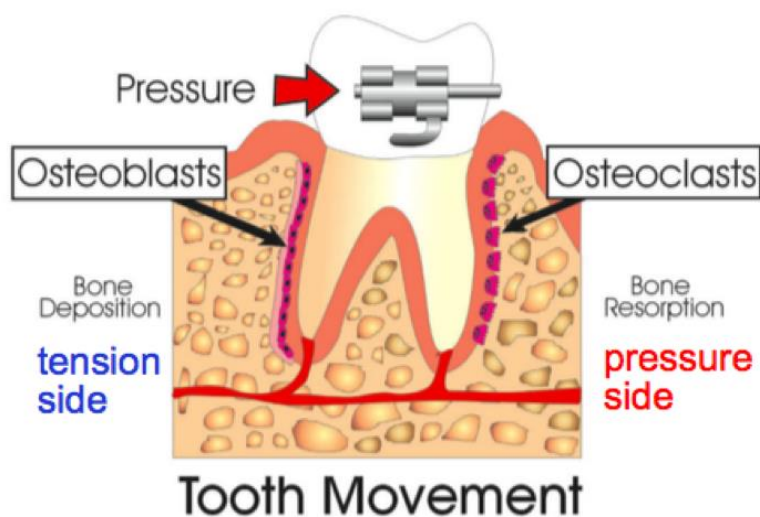
## INTRODUCTION

### 1 Bone Physiology and Orthodontic Tooth Movement

Orthodontic tooth movement (OTM) is made possible by the complex interaction between applied force and biologic response of the bone tissues. Understanding of the intricate mechanisms by which mechanical stimuli are transformed into biologic responses will aid in developing protocols for optimizing orthodontic tooth movement.

Tooth movement is accomplished by a coordinated activity of cells residing in the periodontal ligament (PDL), circulating blood, and the surrounding bone (Figure 1). The pressure-tension theory, the classic theory of OTM, states that chemical signals act as the stimulus for cellular differentiation, resulting in bone remodeling and ultimately tooth movement (Krishnan and Davidovitch, 2006; Schwarz, 1932). Sustained pressure causes the tooth to shift within the PDL space, compressing the ligament on the pressure side and stretching it on the tension side. This leads to fluid expression, as well as decreased perfusion and hypoxia on the compression side while blood flow is maintained or increased on the tension side (Khouw and Goldhaber, 1970).

The combined effect of mechanical stimulus and alterations in the blood flow and oxygen levels create changes in the chemical environment resulting in the release of cytokines, prostaglandins, growth factors and other chemical messengers within a few hours. Animal experiments have revealed that within four hours of sustained force application there is an increase in the nucleotide cyclic adenosine monophosphate (cAMP) (Davidovitch et al., 1972). cAMP acts as a cytosolic second messenger, regulating cellular functions such as differentiation of the local cells in the PDL with the capacity to differentiate to form osteoclasts (bone removing cells) and osteoblasts (bone forming cells) (Roberts, 1989). Studies have also shown that important mediators of cellular response such as prostaglandin, interleukin-1 beta (IL-1 $\beta$ ) and nitric oxide (NO) levels increase within the PDL (Krishnan and Davidovitch, 2006). Furthermore, Kang and colleagues have shown that focal adhesion kinase (FAK) may act as the mechanoreceptor in PDL cells and mediate PGE<sub>2</sub> release (Kang et al., 2010).



**Figure 1. Biological basis of orthodontic tooth movement as explained by the pressure-tension theory**

Upon orthodontic force application, areas of pressure and tension are created as indicated in the schematic. OTM can be explained by two-fold. 1) The PDL is compressed in the pressure side and stretched in the tension side. The mechanical effects on cells of the PDL cause them to release of cytokines, prostaglandins, and other chemical messengers. 2) The change in the PDL space alters the blood flow - it is decreased on compression side and maintained or increased on the tension side. This can create changes in the oxygen levels which then stimulates the release of other chemical messengers. These factors then directly or indirectly stimulate cellular differentiation and activity. Cascade of signaling events eventually lead to activation of osteoclasts on the pressure side leading to bone resorption while osteoblasts form on the tension side to lay down newly formed bone. *(Figure adapted from [www.askanorthodontist.com](http://www.askanorthodontist.com))*

Once differentiated, osteoclasts must remove bone on the compressed side of the PDL while osteoblasts form new bone on the tension side for tooth movement to occur (Figure 1). The cellular activity in the bone surrounding the teeth during orthodontic tooth movement is similar to the cellular activity involved in physiological bone remodeling.

### 1.1 Bone Structure and Function

Bone is a highly specialized and complex connective tissue that constitutes the skeleton. It is lightweight yet strong and serves multiple functions. Bone supports and protects vital organs, provides mechanical stability for body posture, serves as a site for muscle attachment to support locomotion and is involved in the regulation of calcium and phosphate mineral homeostasis (Baron, 2003). In addition, it serves as the primary site of hematopoiesis as it supplies a source of hematopoietic and mesenchymal stem cells (Clarke, 2008).

Bone is composed of a mineralized extracellular matrix containing both organic and inorganic components. The organic portion of bone is primarily composed of collagen type I and make up approximately 35% of bone mass. Type 1 collagen is an elastic protein that provides bone with its tensile strength and flexibility and hence fracture resistance (Baron, 2003). Non-collagenous proteins, such as bone sialoprotein (BSP), osteocalcin (OCN), and osteopontin (OPN), are also present and are thought to regulate the mineralization process (Clarke, 2008). A variety of cells including osteoblasts and osteoclasts also make up a small portion of the organic phase. The inorganic components of bone composes 65% of bone mass and consist of hydroxyapatite crystals  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$  (Hadjidakis and Androulakis, 2006). These crystals pack tightly around collagen in the extracellular matrix, contributing to the hardness and resistance to compressive loading giving bone its rigidity (Clarke, 2008)

The skeleton is composed of two types of bones: an axial skeleton which includes the vertebrae, pelvis, and other flat bones such as the skull and sternum, and an appendicular skeleton which includes all of the long bones (Baron, 2003). Long bones are most bones in the limbs and include the humerus, radius, ulna, femur, tibia and fibula. They are divided into three parts: the epiphysis, metaphysis and diaphysis (Clarke, 2008). The



epiphysis is the portion of the long bone at either end and develops from a center of ossification. It is separated from the rest of the bone by a layer of growth cartilage known as the “physis”. The metaphysis is the zone between the physis and the central portion of the long bone shaft known as the diaphysis (Clarke, 2008).

Anatomically, bone is organized into an external layer of cortical (dense or compact) bone, an internal network of trabecular (spongy or cancellous) bone and bone marrow that resides within the trabecular spaces (Clarke, 2008). The epiphyses and metaphyses are comprised of a trabecular network, while the diaphysis is made of dense compact bone.

Bone is in contact with the soft tissues along two surfaces: an external surface (the periosteal surface) and an internal surface (the endosteal surface). These surfaces are lined with osteogenic cells along the periosteum and the endosteum, respectively (Baron, 2003).

Bone is formed by a process referred to as ossification. During the fetal stage, this occurs by two processes: intramembranous ossification and endochondral ossification (Baron, 2003). Intramembranous ossification involves the formation of bone from connective tissues such as the mesenchyme whereas in endochondral ossification, bone is formed from cartilage (Clarke, 2008). Intramembranous ossification mainly occurs in the flat bones of the skull and also occurs in the mandible, maxilla and clavicle. Endochondral ossification occurs in the long bones and other bones in the body (Baron, 2003; Clarke, 2008).

The alveolar processes of the maxilla and the mandible provide the housing for the dentition. The maxillae consist of two maxilla bones forming the upper jaw and palate of the mouth. It develops entirely by intramembranous ossification and is thought to be ossified from a center of mesenchymal condensation in the maxillary process that appear during the sixth week of prenatal development (Proffit, 2012). Postnatal growth of the maxilla occurs by apposition of bone at sutures that connect maxilla to cranium and cranial base as well as by surface remodeling. The body of the mandible develops from the condensation of mesenchyme just lateral to Meckel’s cartilage and proceeds entirely

by intramembranous bone formation (Proffit, 2012). The condylar cartilage initially develops as a secondary cartilage and fuses with the developing mandibular ramus in the early fetal stages. Both endochondral and intramembranous ossification are important in the growth of the mandible. The surface of the mandibular condyle is covered by cartilage where endochondral changes occur. The rest of the mandible are formed and grows by intramembranous ossification (Proffit, 2012).

## 1.2 Bone Cells and Their Functions

Three major cell-types are found within bone: the bone-resorbing osteoclast, the bone-forming osteoblast and the mechanosensitive osteocyte (terminally differentiated osteoblast) (Baron, 2003). Skeletal development and bone remodelling depend on the communication among the bone cells and the coordinated activity of osteoblasts and osteoclasts referred to as “coupling” (Clarke, 2008).

### 1.2.1 Osteoblasts

Cells of the osteoblast lineage develop from mesenchymal progenitor cells residing in the bone marrow and the periosteum that also give rise to other cell-types including chondrocytes, adipocytes, and fibroblasts (Harada and Rodan, 2003; Minguell et al., 2001; Robling et al., 2006). The major function of mature osteoblasts is to synthesize and secrete osteoid, the organic phase of the bone matrix, and regulate its mineralization (Clarke, 2008). Osteoblast differentiation is characterized by lineage commitment, proliferation, maturation and matrix mineralization. It is regulated by a number of systemic hormones and local factors such as insulin-like growth factors and bone morphogenetic proteins (BMPs). Osteoblast activity is regulated in an autocrine/paracrine manner by these factors whose receptors can be found on osteoblasts (Baron, 2003).

In the last few decades, extensive work has gone into studying osteoblastogenesis from mesenchymal progenitors to a mature bone-forming cell, and the involvement of a number of key transcription factors and transcriptional co-activators have been discovered. These factors function at specific times during osteoblast differentiation and their expression defines the different stages of the osteoblast lineage.

First, the chondrocyte master transcription factor sex-determining region Y (SRY)-box 9 (SOX9) is up-regulated in the mesenchymal progenitors that give rise to either chondrocytes or osteoblasts (Akiyama et al., 2005). The osteoblast master transcription factor runt-related transcription factor 2 (RUNX2) is then expressed and push the proliferating precursor cells to the osteoblast lineage resulting in the formation of osteochondroprogenitors. Subsequent downregulation of SOX9 gives rise to RUNX2-positive osteoprogenitors. (Bendall and Abate-Shen, 2000; Ducky et al., 1997; Robling et al., 2006). At this stage, RUNX2 further mediates expression of osteoblast-specific genes, including collagen type I (COL1) and alkaline phosphatase (ALP), and the upregulation of Osterix (OSX), a second osteoblast master transcription factor (Nishio et al., 2006). In turn, OSX regulates expression of COL1 and bone sialoprotein (BSP) and, together with RUNX2, promotes formation of the preosteoblast also known as immature osteoblast. Further differentiation of the preosteoblast into a mature osteoblast requires the expression of RUNX2, OSX and the canonical Wnt signaling pathway ( $\beta$ -catenin, TCF/LEF1) (Ducky et al., 1997; Glass et al., 2005; Nakashima et al., 2002). The transcription factor ATF-4 also plays an important role in the later stages of osteoblast differentiation. In summary, ATF-4, OSX, and RUNX2 are all required for osteoblast differentiation. In addition, the canonical Wnt transcriptional coactivator  $\beta$ -catenin is also necessary at all stages of osteoblast differentiation.

Aside from the transcription factors, osteoblasts also exhibit differential gene expression specific for the developmental stage. During preosteoblast proliferation, genes required for the activation of proliferation (e.g. *c-Fos*, *c-Jun*, and *c-Myc*) and cell cycle progression (e.g., histones and cyclins) are expressed along with the expression of genes encoding growth factors (fibroblast growth factor [FGF], insulin-like growth factor-1 [IGF-1], transforming growth factor [TGF]), BMPs, cell adhesion proteins (e.g., fibronectin) and COL1 (Feldman, 2013).

Following the initial proliferation, a second stage of gene expression is associated with the maturation and organization of the bone extracellular matrix (ECM). During this stage, collagen synthesis continues and undergoes cross-link maturation. Genes responsible for rendering the extracellular matrix competent for mineralization (e.g. ALP)

are also up-regulated (Feldman, 2013). ALP hydrolyzes ester bonds in organic phosphate compounds under alkaline conditions and is crucial in bone calcification (Anderson, 1989). As such, high ALP activity correlates to extracellular matrix formation in osteoblasts before initiation of mineralization (Gerstenfeld et al., 1987).

Signals for the third stage involve down-regulation of genes involved in proliferation as well as gene expression related to the accumulation of HA in the ECM. Genes encoding non-collagenous proteins with mineral-binding properties (osteopontin (OPN), osteocalcin (OCN) and bone sialoprotein (BSP)) exhibit maximal expression during matrix mineralization (Feldman, 2013). BSP is a glycosylated and sulfated phosphoprotein found almost exclusively in mineralized connective tissues. During bone formation, BSP becomes the nucleus for HA formation (Hunter and Goldberg, 1993). OPN is a highly phosphorylated protein which is a prominent component of the mineralized matrix (Oldberg et al., 1986). OCN is the most abundant non-collagenous protein expressed preferentially by osteoblasts and can bind to calcium ions (Boivin et al., 1990; Wolf, 1996). Active mature osteoblasts then secrete type I collagen and other matrix proteins towards the bone formation surface (Clarke, 2008).

As a row of active osteoblasts secretes unmineralized matrix (osteoid) and advances away from the bone surface, a small number of cells fall behind and become incorporated into the matrix (Clarke, 2008). These osteoblasts begin to generate cytoplasmic processes and become an immature osteocyte. As the matrix matures and mineralizes, the osteocyte begins to mature and express a new set of genes, including those that encode dentin matrix protein-1 (*Dmp1*), matrix extracellular phosphoglycoprotein (*Mepe*), and sclerostin (*Sost*) (Feng et al., 2003; Nampei et al., 2004).

Finally, in mature mineralized cultures, collagenases are elevated, apoptotic activity occurs, and compensatory proliferative activity is evident. This stage appears to serve as editing/remodeling function for modifications in the ECM (Feldman, 2013). At the completion of bone formation, 50 - 70% of osteoblasts undergo apoptosis, with the balance becoming osteocytes or bone-lining cells (Clarke, 2008).

### 1.2.2 Osteoclasts

In contrast to osteoblasts, osteoclasts form by the fusion of mononucleated precursors of the monocyte/macrophage lineage derived from hematopoietic origins (Boyle et al., 2003; Teitelbaum and Ross, 2003). The main function of osteoclasts is bone resorption. Receptor activator of NF-kappa B ligand (RANKL) is critical for osteoclast formation while macrophage-colony stimulating factor (M-CSF) is required for the proliferation, survival, and differentiation of osteoclast precursors, as well as osteoclast survival and cytoskeletal rearrangement required for bone resorption. RANKL and M-CSF are produced in both membrane-bound and soluble forms mainly by marrow stromal cells and osteoblasts (Boyce and Xing, 2008; Boyle et al., 2003; Ross, 2006). Osteoprotegerin (OPG), also produced by stromal cells and osteoblasts, is a decoy protein that occupies RANKL binding sites with high affinity to inhibit its action at the RANK receptor (Boyce and Xing, 2008; Boyle et al., 2003). OPG action on osteoclast precursors and mature osteoclasts prevents new osteoclast generation and induces apoptosis in existing osteoclasts. Hence, the ratio of RANKL and OPG ultimately determines osteoclastogenesis (i.e. higher RANKL:OPG ratio leads to more activation of the RANK receptor and osteoclast maturation) (Blair and Athanasou, 2004).

Osteoclasts are characterized by the presence of multiple nuclei, abundant mitochondria, an extensive Golgi apparatus, and numerous vesicles filled with lysosomal enzymes and vacuoles containing acid phosphatase. The most prominent feature of an actively resorbing osteoclast is its ruffled border with deep plasma membrane folds in the region in contact with the bone matrix (Baron, 2003). Active osteoclasts create an acidic microenvironment within this region via secretion of hydrogen ions to dissolve bone minerals, while secreting various protease and other hydrolases such as cathepsin K to degrade the organic matrix which is mostly composed of type I collagen (Novack and Teitelbaum, 2008).

### 1.2.3 Osteocytes

Osteocytes are terminally differentiated osteoblasts that support bone structure and metabolism (Clarke, 2008). Their function is poorly understood but have been implicated

in mechanosensation, transducing stress signals from bending or stretching of bone into biologic activity of other bone cells (Rubin and Lanyon, 1987). Flow of canalicular fluid in response to external forces induces a variety of responses within osteocytes (Bonewald, 1999). Rapid fluxes of bone calcium across filipodial gap junctions are believed to stimulate transmission of information between osteoblasts on the bone surface and osteocytes within the bone (Plotkin, 2011).

### 1.3 Bone Remodeling

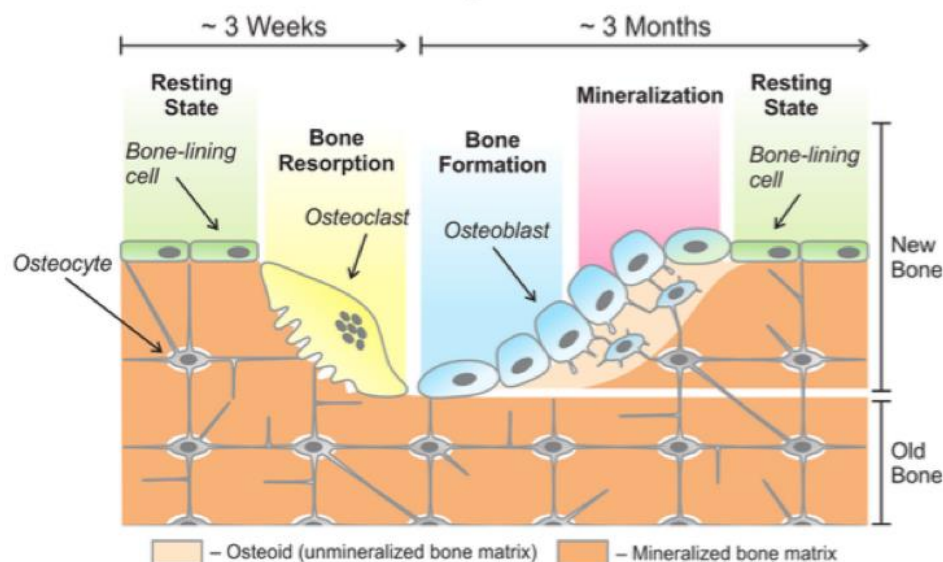
The structural integrity and functionality of bone are maintained throughout life by remodeling, a process that involves the coordinated resorption of old or damaged bone and its replacement with newly mineralized bone matrix. Bone remodeling also plays an important role in maintaining plasma calcium homeostasis (Clarke, 2008). A coordinated balance between osteoclastic bone resorption and osteoblastic bone formation is essential for a healthy and functional skeleton. Under physiological conditions, resorption and formation are tightly coupled, and any imbalance to this process results in a variety of skeletal disorders such as osteoporosis and inflammatory diseases including rheumatoid arthritis and periodontitis (Novack and Teitelbaum, 2008). Bone remodeling is also particularly relevant in orthodontic tooth movement as it is a prerequisite for tooth movement to occur.

The bone remodeling cycle consists of four consecutive phases: activation of osteoclast precursors; resorption, during which osteoclasts digest old bone (2 – 4 week); reversal, when mononuclear cells appear on the bone surface; and formation, when osteoblasts lay down new bone until the resorbed bone is replaced (4 – 6 months) (Baron, 2003; Clarke, 2008). (Figure 2) The regulation of bone remodeling is both systemic and local. The major systemic regulators include parathyroid hormone (PTH), calcitriol, calcitonin and other hormones such as growth hormone, glucocorticoids, and sex hormones. Factors such as insulin-like growth factors (IGFs), prostaglandins (PGs), tumor growth factor- $\beta$  (TGF- $\beta$ ), bone morphogenetic proteins (BMPs), and cytokines are involved as well (Blair and Athanasou, 2004; Boyle et al., 2003). Furthermore, through the RANK/RANKL/OPG system, the processes of bone resorption and formation are tightly coupled allowing bone formation to follow each cycle of bone resorption.

Bone homeostasis is maintained by differential gene expression in bone cells mediated by a specific stimulus mentioned above. Each of these stimuli activates specific intracellular signalling pathways that have been implicated in bone remodeling. Exogenous signals are recognized by cellular receptors found on the cell membrane. Cell membrane receptors are then activated and transfer the information across the cell's cytoplasm to the nucleus through a cascade of intracellular signaling events that involve a complex set of transduction mechanisms. Intracellular receptors bind the stimulus and then translocate that effector to the nucleus where the receptor agonist complex binds to a specific DNA promoter sequence of a gene resulting in the expression of a specific gene.

Signaling pathways implicated in bone formation thus far are: adenosine 3',5'-cyclic monophosphate (cAMP)/protein kinase A (PKA) and MAPK; Wnt/ $\beta$ -catenin;  $\text{Ca}^{2+}$ /NFAT; and PI3K/AKT signaling pathway (Greenblatt et al., 2013; Marie, 2002; Soltanoff et al., 2009). Only the signaling pathways pertinent to this thesis are discussed in the following section.

$\text{Ca}^{2+}$ /NFAT signaling pathway involves stimulation of specific receptors that result in elevations of cytosolic  $\text{Ca}^{2+}$ . This in turn activates calcineurin, an enzyme that dephosphorylates NFATc1-4 transcription factors leading to their nuclear translocation and transcriptional activation (Hogan et al., 2003; Macian, 2005; Rao et al., 1997).  $\text{Ca}^{2+}$ /NFAT pathway has been shown to regulate osteoclastogenesis (Boyle et al., 2003; Novack and Teitelbaum, 2008; Teitelbaum and Ross, 2003) and play important roles in regulation of osteoblast proliferation, differentiation and function (Koga et al., 2005; Sun et al., 2005).



**Figure 2. Bone Remodeling by osteoblasts and osteoclasts**

Bone remodeling is a process that involves the removal of old bone by osteoclasts and its replacement by osteoblasts. It occurs in four phases: activation, resorption, reversal and formation. In response to localized damage to the bone matrix or the actions of systemic hormones, factors are released from osteoblast lineage cells resulting in recruitment of monocyte/macrophage precursors to the bone surface. Differentiation of these precursors leads to formation of a mature osteoclast characterized by its attachment to the bone surface and formation of a specialized apical membrane structure termed the ruffled border. Transport of protons and secretion of hydrolytic enzymes across the ruffled-border membrane causes dissolution of bone mineral and degradation of the organic matrix. The activation and resorption phases take ~3 weeks to complete, and end with death of the osteoclast by apoptosis. During reversal, factors released either from the bone matrix or directly by the osteoclast lead to recruitment and proliferation of mesenchymal progenitor cells at the site of resorbed bone which then differentiate to form osteoblasts. Over the next 3 - 4 months, osteoblasts form bone through secretion of an organic matrix composed primarily of collagen type 1 (COL1), termed osteoid, and regulate its subsequent mineralization. During this process, a number of osteoblasts become embedded within the matrix and terminally differentiate into osteocytes, the most abundant cell-type in bone. (Figure courtesy of Dr. M.W. Grol; PhD Thesis (2013))



The Wnt/ $\beta$ -catenin-dependent canonical signaling pathway also plays an important role in bone formation and remodeling (Robinson et al., 2006). It up-regulates the expression of genes which induce the differentiation and maturation of osteoblast precursor cells and induces an increase in the OPG:RANKL ratio, thus inhibiting osteoclastogenesis (Issack et al., 2008; Kobayashi and Takahashi, 2008; Kubota et al., 2009).

## 2 Mechanotransduction in Bone and Orthodontic Tooth Movement

### 2.1 Mechanotransduction

Mechanotransduction refers to the process by which external mechanical stimuli is translated into biological response. The skeleton remodels in response to physical forces (Wolff's Law) and this process is thought to depend on the detection of mechanical deformation or fluid-shear by osteocytes (Wolff, 1892). Mechanical loading is considered a particularly potent stimulus in eliciting bone remodeling and ultimately leads to a localized increase in the rate of bone remodeling (Robling et al., 2006). While the exact mechanism underlying mechanotransduction is poorly understood, mechanical load-induced strains in the cells and in their extracellular matrix and stresses of fluid flow can mediate changes in gene expression (Robling et al., 2006). When bone is mechanically loaded, bending causes fluid movement in the canalicular network, which leads to shear stress on integrin and actin molecules on the cell surface of osteocytes (Robling et al., 2006). Fluid shear stimulates Adenosine 5-triphosphate (ATP) release which then binds to cell-surface purinergic receptors to trigger a cascade of downstream signaling pathways that ultimately culminates in initiation of osteoclastogenesis and differentiation of osteogenic cells thus promoting bone formation and remodeling (Robling et al., 2006).

Skeletal mechanotransduction makes orthodontic tooth movement possible. Applied orthodontic forces are transmitted to local cells in the periodontal ligament and alveolar bone, stimulating the cells to release secondary signaling molecules that induce pro-inflammatory, angiogenic, and osteogenic properties as described in the previous sections. These, in turn, trigger the process of remodeling of the periodontal ligament and adjacent alveolar bone via gene expression. There is now conclusive evidence that mechanotransduction is mediated by nucleotide release and subsequent purinergic

receptor signaling in bone (Dixon and Sims, 2000; Robling et al., 2006) and this may have important implications for OTM.

## 2.2 Link between Mechanical Stimulation, Nucleotide Signaling and Bone Formation

ATP is present in the cytoplasm of mammalian cells at concentrations of 2 - 5 mM (Orriss et al., 2010). ATP has long been recognized as an intracellular energy source, but it also plays an important regulatory role in a variety of biological processes. ATP is released from cells constitutively and in response to mechanical stimulation, osmotic swelling, shear stress and inflammation (Bodin and Burnstock, 2001; Burnstock, 2007), and is thought to be a key mediator in skeletal cell responses to mechanical stimuli (Dixon and Sims, 2000; Lenertz et al., 2011; Panupinthu et al., 2008; Robling et al., 2006; Robling and Turner, 2009). Smaller amounts are released during neurotransmission and paracrine signalling, while massive amounts are released in response to trauma and cell lysis (Burnstock, 2007). ATP released from stimulated cells trigger intracellular  $\text{Ca}^{2+}$  increases within 1 minute after mechanical loading (Genetos et al., 2005). ATP release from osteoblasts *in vivo* has been reported to range from 1-100  $\mu\text{M}$  (Orriss et al., 2009). Following its release, ATP can act as an extracellular signaling molecule via interactions with specific purinergic receptors to mediate a wide variety of processes. These processes include neurotransmission (Edwards et al., 1992), inflammation (Perregaux and Gabel, 1994), stimulation of secretion (Chan et al., 1995; Chander et al., 1995), regulation of cell proliferation (Weihs et al., 2014), induction of apoptosis (Chow et al., 1997), and bone remodeling (Jones et al., 1997; Morrison et al., 1998). Subsequently, other second messengers such as  $\text{PGE}_2$  (Rawlinson et al., 1991; Reich et al., 1997) and nitric oxide (NO) (Rawlinson et al., 1996) are released from the mechanically stimulated cells and lead to the induction of MAP-kinase signaling (ERK1/2) and *c-Fos* expression (Bowler et al., 1999; Jessop et al., 2002; Lean et al., 1996).

It is now widely accepted that nucleotides released by cells in response to mechanical stimuli act in an autocrine or paracrine fashion to regulate osteoblast and osteoclast function by binding to cell surface purinergic receptors (Buckley et al., 2003; Genetos et al., 2005; Orriss et al., 2009; Romanello et al., 2005). As such, ATP and purinergic receptors are important modulators of both osteoblast and osteoclast activity and hence

the bone remodeling process. The nucleotide induced signaling may help explain the physiological responses of skeletal tissues to mechanical stimuli.

### 3 Purinergic Receptors

Extracellular ATP acts through binding to specific receptors on the cell surface called purinergic receptors. Purinergic receptors are a family of cell surface receptors activated by purines and pyrimidines (ATP, ADP, UTP, UDP, UDP-glucose) and are classified into P1, P2X and P2Y subclasses (Burnstock, 1976; Fredholm et al., 1997). P1 receptors are a family of G protein-coupled receptors activated by extracellular adenosine. P2 receptors are activated by nucleotides and are subdivided into two classes: P2X (ATP-gated cation channels) and P2Y (G protein-coupled receptors) (Burnstock, 2007).

#### 3.1 P2 Receptors

P2 receptors play important roles in regulating cellular physiology and pathology and are implicated in bone remodeling. In mammals, eight P2Y receptor subtypes are known (P2Y<sub>1</sub>, 2, 4, 6, 11-14) whereas seven members of the P2X family (P2X<sub>1</sub>-7) are known to exist (Burnstock, 2007). They are present in a variety of cell types, including osteoblasts and osteoclasts (Burnstock, 2004; Dixon and Sims, 2000; Lenertz et al., 2011; Orriss et al., 2010). In bone, ATP released by mechanical stress (Romanello et al., 2005) binds to P2 receptors on cells of the osteoblast and osteoclast lineages, resulting in remodeling (Buckley et al., 2003; Dixon and Sims, 2000; Lenertz et al., 2011)

The P2X family of receptors are ligand-gated ion channels permeable to Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> (Khakh and North, 2006). P2X receptor channels are formed by three subunits, assembled either as homo- or heterotrimers. Binding of ATP to a P2X receptor causes a conformational change in the shape of the receptor, which results in pore formation and membrane depolarization. Each P2X subunit possesses intracellular carboxyl and amino termini containing consensus binding motifs, transmembrane domains that line the ion pore; and a large extracellular loop that participates in ATP binding (Burnstock, 2007).

#### 3.2 P2X7 Receptors

Among the P2X subclass of receptors, the P2X<sub>7</sub> receptor is unique in its molecular

structure, expression and function (Burnstock, 2007; Jacobson et al., 2002; North, 2002). It is a 595 amino acid polypeptide with two transmembrane domains and a long intracellular C-terminus compared to the other P2X receptors (Denlinger et al., 2001; Surprenant et al., 1996). P2X7 receptors exhibit additional distinctive features from the other P2X receptor family members. These include the requirement of relatively high concentrations of ATP for activation (in the range of 1 mM as compared to  $< 100 \mu\text{M}$  required for other P2X receptors) and 10-30 times more potent activation by 2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP) than by ATP itself (Bianchi et al., 1999; Surprenant et al., 1996). Acute activation of P2X7 receptors via short exposure to ATP rapidly opens a reversible non-selective cation channel within milliseconds allowing the influx of  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$ , and  $\text{K}^{+}$ , while sustained activation leads to the formation of large pores that allow permeation of hydrophilic molecules – up to 900 Da - within seconds (North, 2002). In humans, the P2X7 gene is highly polymorphic and genetic differences within P2X7 affect receptor pore formation and channel function.

P2X7 receptors are highly expressed on immune cells including glial cells, macrophages and other antigen-presenting cells (Surprenant et al., 1996). The presence of P2X7 receptors on immune cells implicates them in immune function and inflammatory responses. Moreover, P2X7 may affect neuronal cells by regulating the processing and release of interleukin-1 $\beta$  (IL-1 $\beta$ ), a key mediator in neurodegeneration, chronic inflammation and chronic pain. Specifically, changes in the intracellular concentration of potassium induce the release of IL-1 $\beta$ , whose active form is a potent pro-inflammatory cytokine (Colomar et al., 2003). Active IL-1 $\beta$  induces a cascade of events including the production of superoxide products (Parvathenani et al., 2003) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (Woolf et al., 1997), both of which have roles in generation or maintenance of pain. The accumulation of intracellular calcium also leads to the release of chemical mediators, such as PGE<sub>2</sub> and IL-1 $\alpha$  and  $\beta$ , all of which have been implicated in inflammation and bone biology (Brough et al., 2003; Gudipaty et al., 2003; Li et al., 2005).

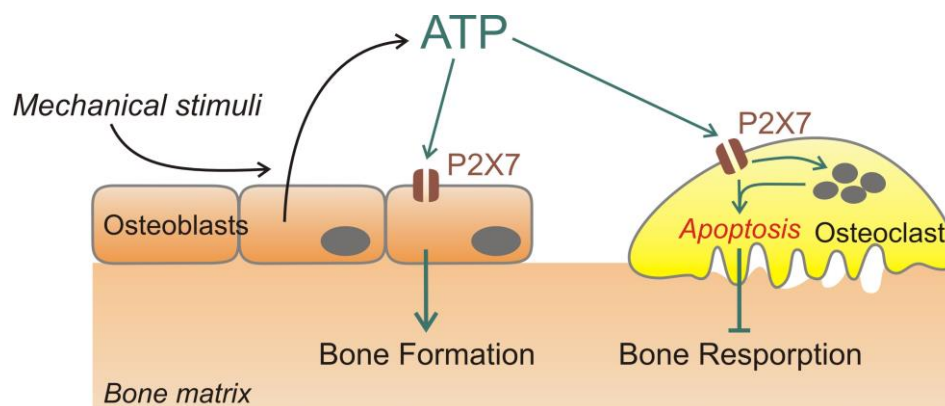
Prolonged activation of P2X7 leads to the formation of large pores, which is followed by cytoskeletal rearrangement such as membrane blebbing, leading eventually to cell death

in immune cells. Considering the overall distribution of P2X7 receptors on pro-inflammatory cells and the functional properties of P2X7 receptors, it is not surprising that many studies have been conducted to determine the role of P2X7 in inflammation. Labasi et al. examined the response of the P2X7 knockout (KO) mice in a monoclonal antibody-induced arthritis model and showed that arthritis severity was significantly attenuated in P2X7 KO mice (Labasi et al., 2002). Moreover, another study by Chessell et al. showed that P2X7-deficient animals did not develop symptoms of pain following a standard induction of inflammatory status (Chessell et al., 2005). Recent findings such as these implicate P2X7 in mediating inflammatory pain. As such, a search for selective antagonists has been pursued by pharmaceutical companies.

### 3.3 P2X7 in Bone Cells

Expression of P2X7 receptors in osteoclasts and osteoblast in rodent and human cells has been consistently reported in a number of publications (Buckley et al., 2003; Burnstock and Verkhatsky, 2009; Gartland et al., 2001; Grol et al., 2009; Nakamura et al., 2000; Orriss et al., 2006; Volonte et al., 2006). However, only a subpopulation of bone derived and calvarial osteoblasts demonstrate a positive nucleotide response (Gartland et al., 2001; Ke et al., 2003; Panupinthu et al., 2008), indicating a possible heterogeneous expression of P2X7 in the cells (Agrawal and Gartland, 2015; Grol et al., 2009).

Growing evidence implicates P2X7 in the induction of osteogenic differentiation of human MSCs (Sun et al., 2013). Sun and colleagues show that ATP release following shockwave treatment led to downstream p38 MAPK activation and *c-Fos*, and *c-Jun* mRNA transcription mediated by P2X7 (Sun et al., 2013). Moreover, shockwave-induced differentiation of MSCs was significantly reduced by the targeted inhibition of P2X7 adding further evidence of a P2X7 mediated effect (Sun et al., 2013). In another study, P2X7 was shown to enhance osteogenic differentiation and mineralization in bone marrow-derived MSC cultures from postmenopausal women (Noronha-Matos et al., 2014). In post-menopausal women, an impaired osteogenic commitment in ageing MSCs is seen as compared to those from younger females. However, osteogenic differentiation



**Figure 3: Consequence of ATP stimulation of P2X7 receptors in osteoblasts vs. osteoclasts**

This general schematic indicates the potential roles for P2X7 receptor signaling in bone. ATP and other nucleotides are released from cells of the osteoblast lineage in response to mechanical stimuli. ATP released into the extracellular environment can act in an autocrine and/or paracrine manner to signal through multiple cell-surface P2 receptors subtypes on osteoblasts and osteoclasts, regulating bone remodeling. P2X7 activation in osteoblasts have been shown to stimulate bone formation, whereas its activation in osteoclasts induces apoptosis resulting in reduction in bone resorption. Thus, the P2X7 receptor may be part of a sophisticated mechanism modulating bone remodeling upon mechanical loading. (*Image reprinted with permission from Purinergic Signalling, Grol et al., 2009; Appendix B*)

and mineralization in postmenopausal MSC cultures were restored by P2X7 activation with BzATP (Noronha-Matos et al., 2014). These findings suggest that P2X7 may indeed promote the differentiation of MSCs into mature osteoblasts, resulting in enhanced bone formation.

Signaling pathways activated by P2X7 receptors have been extensively studied in several cell types (Budagian et al., 2003; Burnstock, 2002; Donnelly-Roberts et al., 2004; Ferrari et al., 1999; Gendron et al., 2003; Humphreys et al., 2000). Activation of P2X7 receptors couples to production of the potent lipid mediator lysophosphatidic acid (LPA) in cells of the osteoblast lineage, resulting in dynamic membrane blebbing and enhanced osteogenesis (Panupinthu et al., 2007; Panupinthu et al., 2008). Activation of phospholipase A2 (PLA<sub>2</sub>) by P2X7 receptors can also lead to synthesis of eicosanoids such as prostaglandins via cyclooxygenase pathways. PLA<sub>2</sub> releases arachidonic acid, the main component of phospholipids of the cell membrane, which can then be metabolized by 2 pathways - the cyclooxygenase pathway involving cyclooxygenase 1 (COX1) and cyclooxygenase 2 (COX2) and the lipoxygenase pathway. COX1 is constitutively found in almost all tissues and is tissue protective. COX2 is activated during inflammatory processes occurring due to cell injury to produce prostaglandins in large amounts. The end products of P2X7 activation, LPA and PGE<sub>2</sub>, have been implicated in mechanotransduction induced by P2X7. However, much of the signalling pathways and downstream events following P2X7 receptor activation in osteoblasts remain to be elucidated.

### 3.4 P2X7 Expression and Polymorphisms in Humans

The P2X7 gene is located on chromosome 12q24.31, spanning 53 kb with 13 exons (Buell et al., 1998). It is highly polymorphic with 40 identified single nucleotide polymorphisms that, in some cases, lead to loss or gain of function mutations (Husted et al., 2013; Wesselius et al., 2011; Wesselius et al., 2013b).

It is hypothesized that variability in the expression of the P2X7 receptor is an important factor in the individual variation to applied orthodontic loads and may provide the key to understanding why some individuals are more prone to external apical root resorption

(Hartsfield, 2009; Vieceilli et al., 2009b). Furthermore, loss-of-function polymorphisms in the P2X7 receptor are associated with lower bone mineral density (Wesselius et al., 2013a), accelerated bone loss (Gartland et al., 2012) and increased osteoporosis risk (Grol et al., 2009; Husted et al., 2013; Jorgensen et al., 2012; Ohlendorff et al., 2007; Wesselius et al., 2013a). More specifically, Ohlendorff et al. reported that 10-year fracture incidence in postmenopausal women is significantly associated with the Glu496Ala and Ile568Asn polymorphisms of the P2X7 receptor (Ohlendorff et al., 2007). The authors also show that the Glu496Ala polymorphism results in decreased susceptibility of osteoclasts to ATP-induced apoptotic death. Impaired osteoclast apoptosis may enhance overall bone resorption, consistent with the increase in fracture incidence and with the skeletal phenotype of the P2X7 KO mouse described by Ke and colleagues (Ke et al., 2003).

### 3.5 P2X7 and Its Role in Mechanotransduction

The role of the P2X7 receptor in osteoblast function has been highly debated. Early reports suggested that P2X7 receptor activation caused enhanced osteoblast apoptosis (Gartland et al., 2001). On the other hand, more recent studies suggest that P2X7 stimulation leads to increased membrane blebbing and bone formation, possibly mediated via increased production of lysophosphatidic acid (LPA) and prostaglandin E2 (PGE<sub>2</sub>) (Li et al., 2005; Panupinthu et al., 2007; Panupinthu et al., 2008). Activation of P2X7 receptors by exogenous nucleotides *in vitro* has been shown to couple to production of LPA and PGE<sub>2</sub>, resulting in increased osteoblast differentiation and matrix mineralization (Li et al., 2005). The P2X7 receptor is also thought to mediate the ERK1/2 activation caused by fluid shear stress in osteoblast-like cells (Liu et al., 2008).

Ke et al. analyzed the long bone skeletal phenotype of P2X7 knockout (KO) mice. In femurs, it was observed that knockout animals had significant reduction in periosteal circumference (bone diameter), but not in length (Ke et al., 2003). This indicates that P2X7 has a role in regulating radial bone growth (periosteal bone formation) and expansion of the bone marrow cavity, but not longitudinal bone growth. In addition, femurs displayed significant reduction in cortical bone content and total bone content. In tibias, trabecular bone displayed an increased number of osteoclasts and a significant



reduction in mass (Ke et al., 2003). This result strongly suggests that the reduction in trabecular bone mass was due to increased osteoclast-mediated resorption. In addition, periosteal bone formation was significantly decreased (Ke et al., 2003).

Interestingly, the skeletal phenotype of a second P2X7 KO mouse model has been described by Gartland and coworkers with contrasting findings (Gartland et al., 2003). These mice showed no overt skeletal phenotype with the exception of thicker cortical bones than wild-type controls. This discrepancy may be due to the presence of a splice variant that escaped deletion in these P2X7 KO mice, resulting in tissue-specific expression of functional P2X7 receptors in these KO mice models (Nicke et al., 2009).

Based on findings from the P2X7 KO mouse described by Ke and colleagues (which have no functional P2X7 receptors), P2X7 receptors appear to be required for normal skeletal growth and anabolic responses to mechanical stimulation as the balance between bone formation and resorption seems to be affected in the knockout animals. Thus, the absence of P2X7 receptor could result in a disturbance in the coordination of bone cell activities (Li et al., 2005; Panupinthu et al., 2008).

### 3.6 Controversy Regarding P2X7 Function in Osteoblasts

Underlying mechanism of P2X7 activation in osteogenesis is still unclear. There are contradicting evidence for the effects of P2X7 activation on osteoblast differentiation and matrix mineralization *in vitro*. Previous studies in our lab have shown that P2X7 activation in rat calvaria-derived osteoblasts lead to osteoblast differentiation and bone formation (Panupinthu et al., 2008). However, a recent study by Orriss et al. reported inhibition of bone formation with P2X7 activation in osteoblasts derived from the same source (Orriss et al., 2012; Orriss et al., 2013). Difference in the culture methodologies between the two groups may explain the discrepancy. Upon investigation into the experimental set-up, we noted a difference in the culture media used to cultivate the cells. Orriss et al. added dexamethasone (10  $\mu$ M) in the growth media in an effort to encourage osteoblast differentiation and bone formation. We believe that dexamethasone may be the reason behind the conflicting findings between the two groups.

#### 4 Steroids and Bone

Corticosteroids are a class of chemicals that includes the steroid hormones produced in the adrenal cortex as well as the synthetic analogues of these hormones. They are involved in a wide range of physiological processes such as stress response, immune and inflammatory response, carbohydrate and protein metabolism, regulation of blood electrolyte levels and behavior (Loeb, 1976; Swartz and Dluhy, 1978).

Glucocorticoids such as cortisol control carbohydrate, fat and protein metabolism, and are anti-inflammatory by preventing phospholipid release, decreasing eosinophil action and a number of other mechanisms. Mineralocorticoids such as aldosterone control electrolyte and water levels, mainly by promoting sodium retention in the kidney. Dexamethasone and its derivatives are almost pure glucocorticoids while prednisone and its derivatives have some mineralocorticoid action in addition to the glucocorticoid effect.

Dexamethasone is a synthetic corticosteroid with anti-inflammatory and immunomodulatory properties against many conditions. Synthetic glucocorticoids are therefore used to treat diseases caused by an overactive immune system, such as rheumatic arthritis, a number of skin diseases, severe allergies, asthma, and even in certain types of cancers among others. They are also used as immunosuppressive medications after organ transplantation. Their anti-inflammatory effect is based on the indirect blocking of PLA<sub>2</sub> and the suppression of the synthesis of both COX1 and COX2 leading to inhibition of prostaglandin and leukotriene synthesis (Coutinho and Chapman, 2011). Their immunosuppressive action is due to the inhibition of interleukins and IFN- $\gamma$  (Coutinho and Chapman, 2011). In dentistry, a low dose of dexamethasone is orally administered to patients before and after wisdom teeth extraction for reduction of post-operative swelling. (Sortino and Cicciu, 2011).

Glucocorticoids exert their effects by binding to the glucocorticoid receptor (GR) in the cytosol. When the glucocorticoids bind to GR, the primary mechanism of action is via regulation of gene transcription. The activated GR complex up-regulates expression of anti-inflammatory proteins in the nucleus and down-regulates the expression of pro-inflammatory proteins by preventing translocation of pro-inflammatory transcription

factors from the cytosol into the nucleus (Rhen and Cidlowski, 2005). The GR is expressed in almost every cell in several forms. It induces many different effects in different part of the body, which renders glucocorticoid its pleiotropic effects including many harmful side effects.

Long-term use of corticosteroids has been associated with a number of side effects such as thrush, bone loss, cataracts, easy bruising or muscle weakness (Buchman, 2001). A serious adverse effect worth noting is glucocorticoid-induced osteoporosis. Glucocorticoids are involved in bone physiology but the exact mechanism is unclear. It has been shown that osteoblasts and osteoclasts express glucocorticoid receptors and this expression is affected by pro-inflammatory factors such as IL-6 and IL-11 (Angeli et al., 2002). Glucocorticoids increase bone resorption by stimulating osteoclastogenesis by shifting the RANKL:OPG ratio (Hofbauer et al., 1999) and by stimulating collagenase 3 post-transcriptionally (Knauper et al., 1996). It has also been demonstrated that glucocorticoids inhibit new bone formation by directly inhibiting intestinal calcium absorption and indirectly affecting the level of sex hormones. Glucocorticoids have been shown to reduce estrogen levels and increase parathyroid hormone levels in the blood leading to decreased bone formation (Canalis and Delany, 2002). In addition, the expressions of paracrine factors such as insulin-like growth factor (IGF-I) and growth hormone are also influenced (Heck et al., 1997). The changes in hormone levels and bioactive molecules closely associated with bone metabolism seem to result in a disturbance in bone homeostasis. Glucocorticoid induced inhibition of new bone formation and stimulation of bone resorption leads to bone dysplasia such as osteoporosis (Olney, 2009).

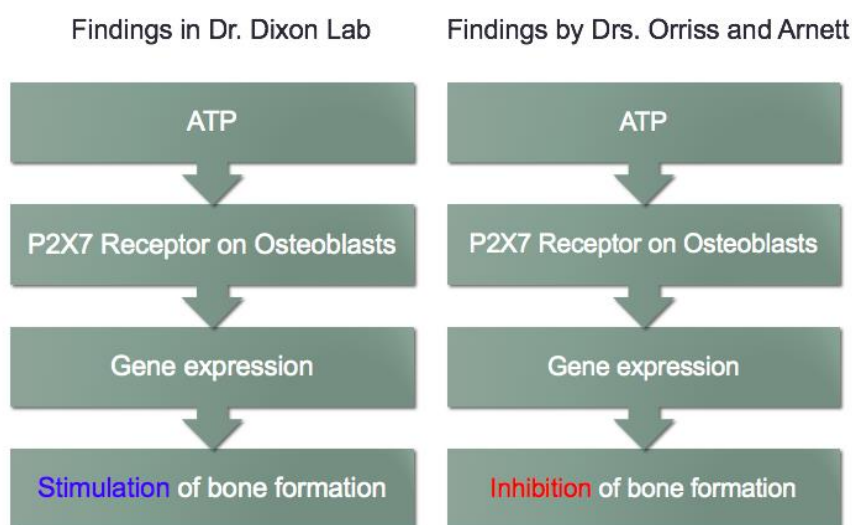
A few studies have looked at the effects of glucocorticoids and OTM. However, the difference in the experimental design such as the animal model, dosages, induction periods, and the relative anti-inflammatory activity of the glucocorticoids seem to create discrepancy in the findings (Bartzela et al., 2009). In rabbits, cortisone administration was shown to result in a significant increase in the rate of OTM. The relapse rate was also faster in the treatment group as compared to the control (Ashcraft et al., 1992). In rats, prednisolone was shown to have no significant effect on the rate of tooth movement

while a similar study done with methylprednisolone show increase in the rate of tooth movement (Ong et al., 2000). Kalia and colleagues looked at the response of rat alveolar bone upon acute and chronic methylprednisolone administration with or without orthodontic forces (Kalia et al., 2004). Acute corticosteroid treatment led to a reduction in bone turnover but the rate of tooth movement was unaffected. In the chronic group, the degree of resorption was doubled which led to an increased rate of tooth movement. The authors conclude that with chronic glucocorticoid use, the rate of active tooth movement is greater, however this results in unstable final tooth position (Kalia et al., 2004). They suggest that it is possible to treat patients on corticosteroid therapy with some adjustments. For patients with short-term administration of the drug, it may be best to postpone orthodontic treatment until the chronic phase is over and the patient is off the medication. If the patient is already undergoing orthodontics, appliance adjustments should be kept at minimal and the appointments should be scheduled with longer intervals as bone turnover will likely be delayed and OTM could take longer in the acute phase of glucocorticoid therapy. For patients undergoing long-term drug therapy, the rate of tooth movement will be increased and the orthodontic appliance can be controlled as usual or more frequently. It may also be necessary to decrease the amount of force used. Furthermore, once the patient is in the retention phase of orthodontic treatment, a more rigorous retention protocol may be recommended (Kalia et al., 2004).

## 5 Rationale, Hypotheses and Objectives of the Research

Given the importance of purinergic signaling in osteoblast differentiation and function, the objective of this project was to investigate the signal transduction pathways that function downstream of P2X7 in cells of the osteoblast lineage. As described above, there is a controversy regarding the consequences of P2X7 receptor activation in osteoblasts. In this study, we investigated the downstream events that follow BzATP activation of P2X7, specifically in gene expression. Considering the important role of P2X7 plays in skeletal mechanotransduction, we hypothesized that P2X7 activation induces the expression of anabolic genes involved in bone formation in osteoblasts. Furthermore, we hypothesized that this anabolic effect of P2X7 on gene expression is inhibited by glucocorticoids. In the present study, we assessed effects of the glucocorticoid dexamethasone (Figure 4).

Target genes of interest are those implicated in osteogenesis such as transcription factors responsible for osteoblast proliferation and differentiation as well as genes that encode bone matrix proteins.



**Figure 4. Controversy regarding the consequences of P2X7 receptor activation in osteoblasts**

This general schematic summarizes the current controversy surrounding the exact role P2X7 plays in osteoblast function and eventual bone formation. Left panel indicates the findings from past lab members of Dr. Dixon's laboratory. Observations from Dr. Dixon's lab have consistently shown stimulation of bone formation with ATP-induced P2X7 activation in osteoblasts (Panupinthu et al., 2008; Grol et al., 2009). Right panel shows the observations of a U.K. group led by Dr. Arnett. In contrast to the findings from our lab, they have shown that ATP activation of P2X7 in osteoblasts leads to inhibition of bone formation. Differences observed may be attributed to the differences in the culture condition used by the two groups.

## MATERIALS AND METHODS

### 1 Materials and Solutions

$\alpha$ -Minimum essential medium ( $\alpha$ -MEM), heat-inactivated fetal bovine serum (FBS), antibiotic solution (10,000 U/ml penicillin, 10,000 mg/ml streptomycin, and 25 mg/ml amphotericin B), trypsin solution, Dulbecco's phosphate buffered saline (DPBS) were obtained from GIBCO (Life Technologies Inc., Burlington, ON, Canada). TRIzol reagent and UltraPure distilled water (DNase/RNase-free) were obtained from Invitrogen (Life Technologies). RNeasy Mini Kit was from QIAGEN (Toronto, ON, Canada). qScript XLT One-Step RTqPCR Toughmix ROX was purchased from Quanta. Primers and probes for *Ptgs2* (COX2, Mm00478374\_m1), *Dmpl* (Mm01208363\_m1), *c-Fos* (Mm00487425\_m1), *Mepe* (Mm\_02525159\_s1), *Colla1* (Mm00801666\_g1), *Bglap* (osteoclastin; Mm03413826\_mH), *c-Myc* (Mm00487804\_m1), *Mmp9* (Mm00442991\_m1), *Alpl* (alkaline phosphatase; Mm01187113\_g1) and 18S rRNA were obtained from Applied Biosystems (Life Technologies). 2'-3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate triethylammonium salt (BzATP) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2 Cell Culture

The MC3T3-E1 osteoblast precursor cell line (subclone 4) was obtained from the American Type Culture Collection (Rockville, MD, USA). MC3T3-E1 cells were subcultured twice weekly and maintained in  $\alpha$ -MEM, supplemented with 10% FBS and 1% antibiotic antimycotic solution (culture medium) at 37°C and 5% CO<sub>2</sub>.

### 3 RNA Isolation

Total RNA was extracted using TRIZOL® reagent (Invitrogen, Paisley, UK) and RNeasy according to the manufacturer's instructions. Total RNA was quantified spectrophotometrically by measuring absorbance at 260 nM. RNA was diluted to 25ng/ $\mu$ l in RNase free water, aliquoted and stored at -80 °C until amplification by Real-Time RT-PCR.

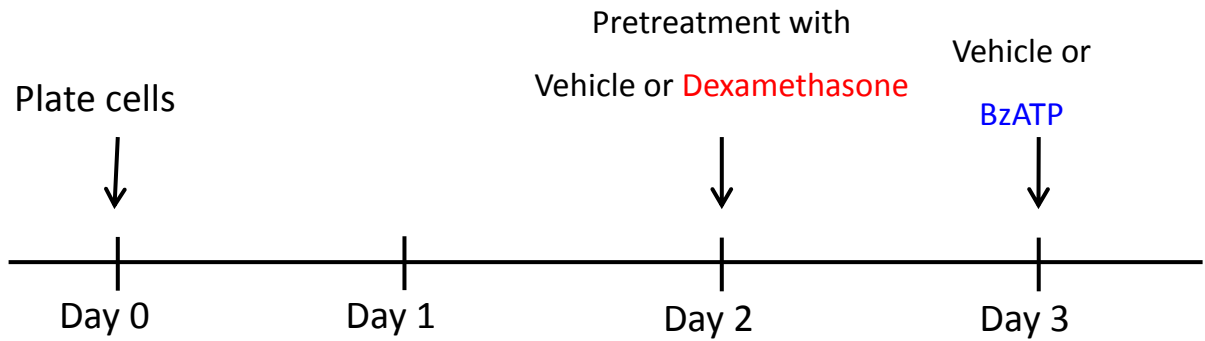
#### 4 Real-Time RT-PCR Analyses

MC3T3-E1 cells (mouse calvarial preosteoblasts) were plated at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup> on Falcon 6-well plates in culture medium. After 2 days, cells were placed in serum-free medium and incubated overnight. Select wells were pre-treated with dexamethasone (100 nM) or vehicle (100% ethanol) overnight (Figure 5). On the day of the experiment, cells were incubated with BzATP (300  $\mu$ M) or vehicle (divalent cation free buffer, DCFB) for the indicated times and total RNA was isolated as mentioned above. Real-time PCR was performed using the ABI Prism 7900 HT Sequence Detector (PerkinElmer) with 15  $\mu$ l final reaction volumes containing 25 ng RNA sample, qScript XLT One-Step RTqPCR Toughmix, and one of *Ptgs2*, *c-Fos*, *Mepe*, *Dmpl*, *Colla1*, *Bglap*, *Mmp9*, *c-Myc*, *Alpl* or 18S rRNA primers and probes. Reverse transcription was performed at 50 °C for 10 min followed by 40 cycles of amplification at an annealing temperature of 60 °C. Reactions for each sample were performed in triplicate. All samples were normalized to 18S rRNA, and time zero or vehicle- treated controls using the delta-delta cycle threshold (DDC<sub>t</sub>) method.

#### 5 Data Analysis and Statistical Analyses

Data are shown as means  $\pm$  standard error of the mean (S.E.M) for the number (*n*) of experiments indicated, each performed in triplicate. Differences among three or more groups were evaluated by two-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* multiple comparisons test. Differences were accepted as statistically significant at  $p < 0.05$ . Data analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).





**Figure 5. Timeline of the experiments**

MC3T3-E1 cells were plated at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup> in Falcon 6-well plates in culture medium (day 0). After 2 days, cells were placed in serum-free medium and incubated overnight with dexamethasone (100 nM) or vehicle (ethanol). On the day of the experiment (day 3), cells were incubated with BzATP (300  $\mu$ M) or vehicle (DCFB) at time 0 hours. Total RNA was then isolated using Trizol at 0, 0.5, 1, 3 and 6 hours.

## RESULTS

### 1 BzATP stimulation of *Ptgs2* (COX2) was inhibited by dexamethasone

*Ptgs2* is the gene that encodes prostaglandin-endoperoxide synthase 2 also known as cyclooxygenase 2 (COX2), a critical enzyme for the synthesis of prostaglandin E2 (PGE<sub>2</sub>). Recent studies suggest that a number of effects of P2X7 receptor activation during osteoblast mechanotransduction may be mediated via prostaglandins (PGs) such as PGE<sub>2</sub> (Li et al., 2005).

Changes in *Ptgs2* (COX2) expression were examined in MC3T3-E1 cells following treatment with vehicle (DCF Buffer) or BzATP (300  $\mu$ M) in the presence or absence of dexamethasone (100 nM). As previously shown by our lab (Grol et al., 2013), BzATP induced significant stimulation of *Ptgs2* expression ( $120 \pm 46$  fold difference) as compared to the vehicle-treated cells. *Ptgs2* expression reached the peak at 1 h and returned to basal levels by 6 h. Statistically significant differences were present between vehicle treated cells vs BzATP treatment at 1 h and 3 h ( $p < 0.001$  for both time points) whereas dexamethasone alone had no effect on *Ptgs2* expression (Figure 6). Dexamethasone, however, significantly reduced BzATP induced *Ptgs2* expression at 1h and 3 h ( $P < 0.001$  for both time points) (Figure 6).

### 2 BzATP stimulation of *Dmp1* was completely inhibited by dexamethasone

*Dmp1* encodes dentin matrix acidic phosphoprotein 1, which is found in the extracellular matrix of bone, dentin and cementum as well as in other non-mineralized tissues (George et al., 1993; MacDougall et al., 1998). *Dmp1* has been shown to be critical for the mineralization of bone and dentin (Feng et al., 2006). Bone cells such as osteocytes, osteoblasts and hypertrophic chondrocytes express *Dmp1* (Feng et al., 2003; Toyosawa et al., 2012).

We report, for the first time, that BzATP stimulates *Dmp1* expression, which peaked at 3 h post BzATP treatment with a maximum fold difference of  $8.4 \pm 2.9$ . Further, this was statistically significant at 1 h and 3 h ( $p < 0.01$ ;  $p < 0.001$ , respectively). Dexamethasone

completely abolished the BzATP-induced *Dmp1* expression (Figure 7;  $p < 0.01$  for 1 h,  $p < 0.001$  for 3 h).

### 3 BzATP dramatically stimulated *c-Fos* with no inhibition by dexamethasone, indicating specificity

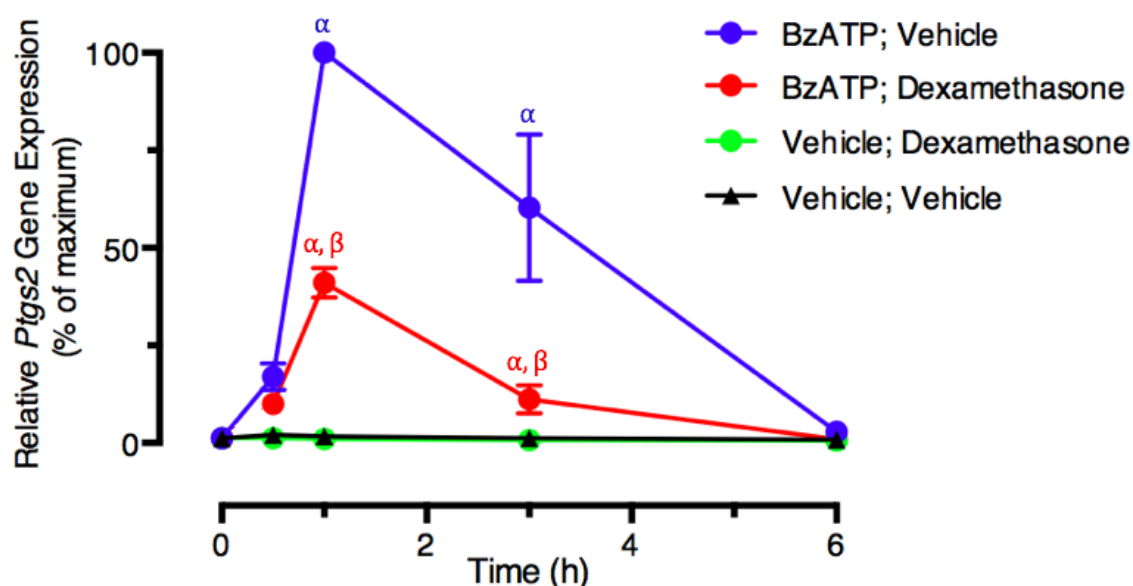
*c-Fos* is an immediate early gene, whose activation can be linked to different signaling cascades responsible for cell proliferation, differentiation and survival (Dixon and Sims, 2000). *c-Fos* plays a crucial role in regulating proliferation and differentiation of bone and cartilage cells (Hipskind and Bilbe, 1998). Fos proteins are highly expressed in osteoblast precursors, but their levels decline following the initial proliferation stage (McCabe et al., 1995).

We confirmed a dramatic increase in *c-Fos* expression at an early time point (30 min) with BzATP stimulation of P2X7 (Figure 8;  $p < 0.001$  for 0.5 h and  $p < 0.001$  for 1 h). However, in contrast to the findings for *Ptgs2* and *Dmp1*, dexamethasone did not inhibit this stimulatory effect indicating specificity of dexamethasone action on the expression of particular genes (Figure 8).

### 4 BzATP and dexamethasone did not affect *Colla1* expression

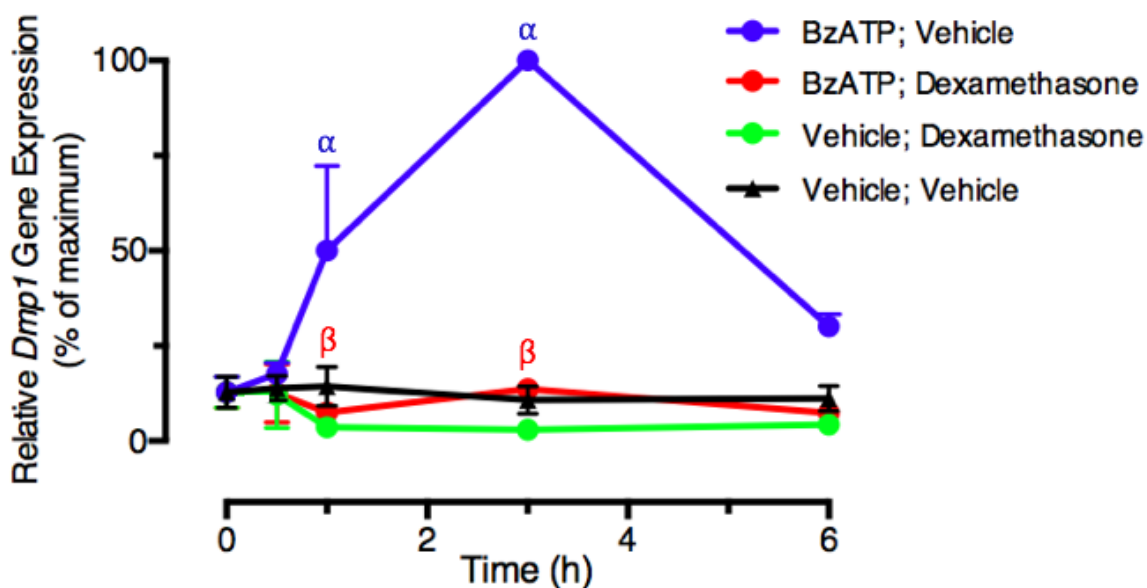
We also looked at *Colla1*, which encodes collagen type 1 alpha 1 found in connective tissues such as bone, tendon and skin. Throughout the 6-hour time course, there were no significant effects of BzATP or dexamethasone on *Colla1* expression (Figure 9). The same was found for the following genes: *Bglap* (encoding osteocalcin), *Alpl*, *c-Myc*, and *Mmp9*. This does not rule out the possibility of late induction of these genes following P2X7 activation since, in the present study, the time course was limited to 6 hours.

NB: Please refer to Appendix A for data presented as relative gene expression without normalization as percentage of the maximum value.



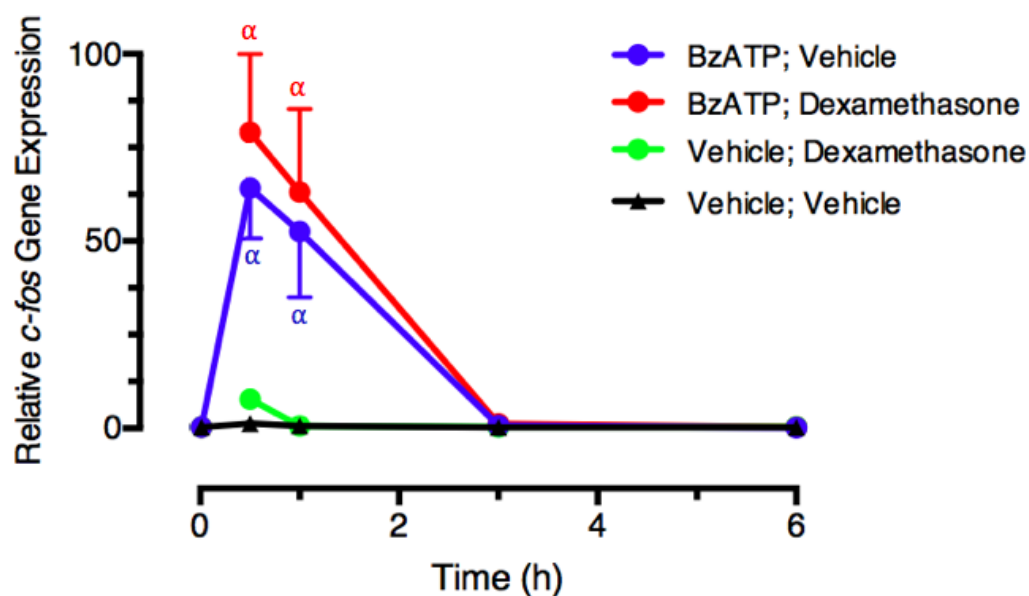
**Figure 6: Dexamethasone suppresses P2X7-induced expression of *Ptg2***

MC3T3-E1 cells were pretreated with vehicle or dexamethasone (100 nM) overnight and then treated with vehicle or BzATP (300  $\mu$ M) at time 0. Total RNA was isolated at the indicated times. RT-PCR was performed to assess expression levels of *Ptg2*. Data were normalized to levels of 18S ribosomal RNA, and relative to values for vehicle-treated cultures at time 0. Data are shown as percentages of the maximum value in each individual experiment and are presented as means  $\pm$  S.E.M. ( $n = 3$  independent experiments, each performed in triplicate). The mean maximum value (100%) for these 3 experiments was  $120 \pm 46$  fold increase relative to vehicle-treated cultures at time 0. Differences were evaluated by two-way analysis of variance followed by a Bonferroni multiple comparisons test.  $\alpha$  indicates significant difference from vehicle/vehicle at each time point;  $\beta$  indicates significant effect of dexamethasone pretreatment ( $p < 0.05$ ). The following points were also significantly different than vehicle/vehicle at time 0 – BzATP/vehicle at 1h and 3 hrs; BzATP/dexamethasone at 1h.



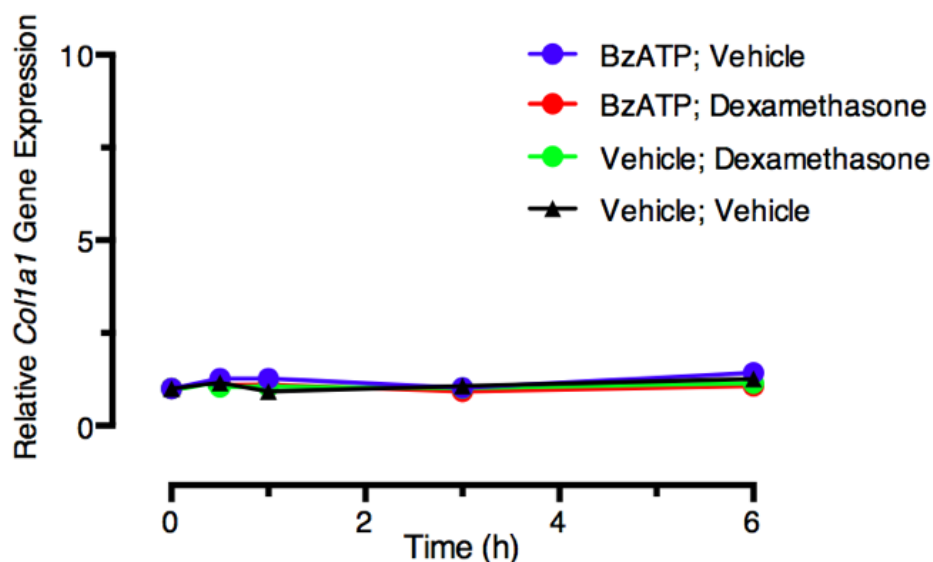
**Figure 7: BzATP stimulation of *Dmp1* is completely inhibited by dexamethasone**

MC3T3-E1 cells were pretreated with vehicle or dexamethasone (100 nM) overnight and then treated with vehicle or BzATP (300  $\mu$ M) at time 0. Total RNA was isolated at the indicated times. RT-PCR was performed to assess expression levels of *Dmp1*. Data were normalized to levels of 18S ribosomal RNA, and relative to values for vehicle-treated cultures at time 0. Data are shown as percentages of the maximum value in each individual experiment and are presented as means  $\pm$  S.E.M. ( $n = 3$  independent experiments, each performed in triplicate). The mean maximum value (100%) for these 3 experiments was  $8.4 \pm 2.9$  fold increase relative to vehicle-treated cultures at time 0. Statistical analysis was performed as mentioned in methods.  $\alpha$  indicates significant differences between BzATP treatment and vehicle at each time point;  $\beta$  indicates significant differences between BzATP treatment vs. BzATP + Dexamethasone pretreatment. ( $p < 0.05$ ). The following points were also significantly different than vehicle/vehicle at time 0 – BzATP/vehicle at 1h and 3 hrs.



**Figure 8: BzATP stimulates *c-Fos* expression with no inhibition by dexamethasone**

MC3T3-E1 cells were pretreated with vehicle or dexamethasone (100 nM) overnight and then treated with vehicle or BzATP (300  $\mu$ M) at time 0. Total RNA was isolated at the indicated times. RT-PCR was performed to assess expression levels of *c-Fos*. Data were normalized to levels of 18S ribosomal RNA, and relative to values for vehicle-treated cultures at time 0. Data are shown as percentages of the maximum value in each individual experiment and are presented as means  $\pm$  S.E.M. ( $n = 3$  independent experiments, each performed in triplicate). The mean maximum value (100%) for these 3 experiments was  $279 \pm 48$  fold increase relative to vehicle-treated cultures at time 0. Statistical analysis was performed as mentioned in methods.  $\alpha$  indicates significant differences from vehicle at each time point; No significant differences between BzATP treatment vs. BzATP + dexamethasone pretreatment was found. ( $p < 0.05$ ). The following points were also significantly different than vehicle/vehicle at time 0 – BzATP/vehicle at 1h and 3 hrs; BzATP/dexamethasone at 1h and 3 hrs.



**Figure 9: BzATP and dexamethasone do not affect *Col1a1* expression up to 6 hours**

MC3T3-E1 cells were pretreated with vehicle or dexamethasone (100 nM) overnight and then treated with vehicle or BzATP (300  $\mu$ M) at time 0. Total RNA was isolated at the indicated times. RT-PCR was performed to assess expression levels of *Col1a1*. Data were normalized to levels of 18S ribosomal RNA, and relative to values for vehicle-treated cultures at time 0. Data are shown relative gene expression and are presented as means  $\pm$  S.E.M. ( $n = 3$  independent experiments, each performed in triplicate). Statistical analysis was performed as mentioned in methods. No statistically significant differences were found between the treatment groups.

## DISCUSSION

The P2X7 receptor plays a role in the regulation of osteogenesis and is required for a proper skeletal response in mechanical loading. This has important implications in orthodontics, as OTM is dependent on mechanotransduction within the alveolar bone.

The exact role P2X7 plays in osteoblast activation and function and hence osteogenesis remains poorly understood. Currently, there is contradicting evidence for the effects of P2X7 activation on osteoblast differentiation and bone formation *in vitro*. While Orriss et al. showed inhibition of bone formation with P2X7 activation in rat calvarial osteoblasts (Orriss et al., 2012), a previous study by our lab showed an opposite effect of increased osteoblast differentiation and bone formation following P2X7 activation in the cell-line derived from the same animal model (Panupinthu et al., 2008).

Orriss et al. attribute their findings to a P2 receptor-dependent and/or receptor independent mechanism via hydrolysis of extracellular nucleotides to pyrophosphate, a known inhibitor of mineralization (Orriss et al., 2012). In a follow up study, they further strengthen their argument by showing that the negative effect of ATP on bone formation was reversed with apyrase treatment (which breaks down ATP) (Orriss et al., 2013). This suggests that nucleotide signaling via purinergic receptors may potentially induce an anti-osteogenic mechanism (Orriss et al., 2013).

Results from our lab provide evidence of a possible coupling of P2X7 activation with production of potent lipid mediators, which may have an osteogenic effect. Panupinthu et al. show that P2X7 activation of osteoblast function may involve phospholipase D and phospholipase A2 stimulation leading to the production of LPA and PGE<sub>2</sub>, which contribute to enhanced osteogenesis (Panupinthu et al., 2007; Panupinthu et al., 2008). LPA signaling involves Rho-associated kinase, which has established roles in driving non-committed cells towards osteoblast lineage (McBeath et al., 2004). In addition, Grol et al. showed that Ca<sup>2+</sup> influx following P2X7 activation causes a sustained increase in metabolic acid efflux (measurement of osteoblast cellular activity) dependent on glucose and phosphatidylinositol 3-kinase activity (Grol et al., 2012). Furthermore, the ATP



induced  $\text{Ca}^{2+}$  signalling in osteoblasts is dose and duration dependent and is transduced to distinct cellular signals (Grol et al., 2013). Low concentrations of ATP (1 nM to 100  $\mu\text{M}$ ) act through P2Y receptors to elicit transient  $\text{Ca}^{2+}$ -NFAT (nuclear factor of activated T-cells) signaling whereas high ATP concentrations (300  $\mu\text{M}$  to 10 mM) act through P2X7 to induce a more sustained  $\text{Ca}^{2+}$ -NFAT signaling ultimately stimulating expression of *Ptgs2* (COX2) (Grol et al., 2013). This up-regulation of *Ptgs2*(COX2) may translate to an increased production of  $\text{PGE}_2$ .

Further evidence supporting positive role of P2X7 in osteoblasts include the observations of reduced alkaline phosphatase activity in osteoblasts isolated from P2X7 KO rats (Panupinthu et al., 2008), decreased periosteal bone formation in long bones of P2X7 KO mice (Ke et al., 2003), and their reduced osteogenesis in response to mechanical loading (Li et al., 2005). In addition, several polymorphisms in the P2X7 leading to reduced function of the P2X7 are associated with increased risk of osteoporosis in humans (Gartland et al., 2012; Jorgensen et al., 2012; Ohlendorff et al., 2007).

The discrepancy observed between the findings of Orriss et al. and our lab may be attributed to the differences in the experimental set up. Upon review of the culture conditions, we noted several differences: a) dexamethasone was added to the culture media used by Orriss et al. due to that fact that glucocorticoids have been shown to be osteogenic *in vitro* (Bellows et al., 1994) b) duration of culture period, and c) the age of the animals at the time cells were isolated. Given the pleiotropic nature of glucocorticoid action in bone, we suspect that dexamethasone may be the reason behind the contradicting observations.

In this study, we examined the changes in gene expression triggered by BzATP activation of endogenous P2X7 receptors in osteoblasts. Furthermore, we examined the effect dexamethasone has on BzATP induced gene expression in an effort to clarify the mechanism of action dexamethasone has on osteoblast differentiation and function.

*Ptgs2* is the gene that encodes prostaglandin-endoperoxide synthase 2 also known as cyclooxygenase 2 (COX2), a critical enzyme for the synthesis of prostaglandin E2 ( $\text{PGE}_2$ ). Recent studies suggest that a number of effects of P2X7 receptor activation

during osteoblast mechanotransduction may be mediated via prostaglandins (PGs) such as PGE<sub>2</sub> (Li et al., 2005).

Prostaglandins are important mediators of mechanical stress (Blackwell et al., 2010). Other stimulants such as growth factors (Platelet-derived growth factors [PDGF]), hormones (parathyroid hormone [PTH]), and interleukins and other cytokines also induce PGE<sub>2</sub> production and affect bone remodeling and tooth movement (Blackwell et al., 2010). Clinical and animal studies have demonstrated the role of PGs in modulating bone remodeling by stimulating bone resorption and bone formation. PGs increase osteoclast number as well as their capacity to form a ruffled border resulting in subsequent increase in bone resorption (Klein, 1970). In orthodontics, orthodontic force application induces the synthesis of PGs, which in turn stimulate osteoclastic bone resorption (Davidovitch et al., 1988; Yamasaki et al., 1984). A local injection of PGs into the paradental tissues of rodents resulted in an increase in osteoclast numbers (Yamasaki et al., 1984). Moreover, a reduced rate of tooth movement was observed after the administration of indomethacin, an anti-inflammatory agent which inhibits PG synthesis by blocking COX1 and COX2 (Chumbley and Tuncay, 1986). The exact mechanistic role of PGs in bone resorption is not clear but it is thought to stimulate cells to produce cyclic adenosine monophosphate, which is an important chemical messenger for bone resorption (Klein, 1970; Raisz and Koolemans-Beynen, 1974). In osteoblastic cells, PGE<sub>2</sub> stimulates their differentiation and eventual new bone formation, coupling bone resorption *in vitro* (Blackwell et al., 2010). Recent work from our lab has shown that Ca<sup>2+</sup>-NFAT signaling pathway stimulates expression of *Ptgs2* (COX2) downstream of the P2X7 receptor in osteoblasts within 3 hours of ATP treatment (Grol et al., 2013) further suggesting that PGE<sub>2</sub> produced by the activation of P2X7 may act as an autocrine factor for osteoblast activation.

Given the important role of COX2 and PGE<sub>2</sub> in osteoblast differentiation and responses to mechanical stimuli (Blackwell et al., 2010), we examined changes in *Ptgs2* expression following ATP activation of P2X7. We confirm that BzATP stimulation of P2X7 leads to a significant increase in *Ptgs2* expression at similar time points as observed in our previous study (Grol et al., 2013). Furthermore, we show, for the first time, that BzATP induced *Ptgs2* expression is inhibited markedly by dexamethasone (Figure 6). This

inhibitory action of dexamethasone on *Ptgs2* gene expression may help explain the current controversy surrounding the exact role of P2X7 activation in osteoblasts. Moreover, this may also help deduce the mechanism of action of glucocorticoids in bone cells at a transcriptional level.

Interestingly, we show that dexamethasone's inhibitory effect on *Ptgs2* was incomplete as there was evidence of some *Ptgs2* expression. This may be explained by multiple factors. First, the extent of inhibition may be dependent on the concentration of dexamethasone in a dose-dependent manner. It is possible that we would see greater inhibition with a dose larger than 100 nM as used in this study. Secondly, we cannot rule out the involvement of other signaling pathways or other P2 receptors whose signaling pathways may not be sensitive to dexamethasone.

*Dmp1* gene encodes dentin matrix acidic phosphoprotein 1, an extracellular matrix protein that belongs to the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family of proteins (Staines et al., 2012). Dentin matrix acidic phosphoprotein 1 is found in the extracellular matrix of bone, dentin and cementum as well as in other non-mineralized tissues (George et al., 1993; MacDougall et al., 1998). *Dmp1* is critical for the mineralization of bone and dentin, as mutations in the gene are known to cause autosomal recessive hypophosphatemia, which can manifest as rickets and osteomalacia (Feng et al., 2006). In bone, *Dmp1* is expressed in osteocytes, osteoblasts and hypertrophic chondrocytes (Feng et al., 2003; Toyosawa et al., 2012).

In this study, a significant stimulation of *Dmp1* expression with BzATP is also observed, although not to the same fold difference as seen with *Ptgs2* ( $120 \pm 46$  [*Ptgs2*] vs  $8.4 \pm 2.9$  [*Dmp1*]), (Figure 7). *Dmp1* expression peaks at a later time point than *Ptgs2* which may be due to the fact that the genes required for the matrix proteins are activated downstream of *Ptgs2*. Interestingly, there was a complete inhibition of *Dmp1* stimulation by dexamethasone. In contrast to *Ptgs2*, the signaling pathway that leads to *Dmp1* expression may be highly dependent on P2X7 and selectively inhibited by dexamethasone.

As mentioned briefly in the introduction, extracellular stimuli regulate gene expression via the activation of intracellular signaling systems that transduce the signal from membrane-bound receptors to transcription factors. This results in the rapid, transient induction of genes known as “immediate early gene” whose products mediate the appropriate cellular response. *c-Fos* is an example of an immediate early gene, whose activation can be linked to different signaling cascades targeting distinct promoter elements through the phosphorylation of transcription factors (Dixon and Sims, 2000). Fos proteins are highly expressed in osteoblast precursors, but their levels decline following the initial proliferation stage (McCabe et al., 1995).

*c-Fos* encodes a transcription factor which, as part of an activator protein 1 (AP-1) complex, plays a crucial role in regulating proliferation and differentiation of bone and cartilage cells (Hipskind and Bilbe, 1998). *c-Fos* induction plays an important role *in vitro* in driving immortalized fibroblasts to enter the cell cycle and plays an important role *in vivo* in the skeletal system (Bowler et al., 1999). Mice overexpressing *c-Fos* develop osteosarcomas and chondrosarcomas, whereas mice lacking *c-Fos* fail to develop osteoclasts and thus develop osteopetrosis in which the bone remodeling process becomes shifted towards bone accumulation (David et al., 2005; Johnson et al., 1992; Wang et al., 1992). These findings implicate that *c-Fos* plays a key role in the regulation of skeletal cells. Accordingly, a number of proteins found in differentiating bone cells have regulatory AP-1 sites in their promoters, and a variety of extracellular factors involved in osteogenesis activate *c-Fos* transcription in cultured bone cells *in vitro* (Evans, 1996; Hipskind and Bilbe, 1998).

It has previously been shown by our lab that extracellular ATP rapidly increases *c-Fos* mRNA in UMR-106 osteoblastic cells at levels as low as 10  $\mu$ M via P2 receptors (Dixon and Sims, 2000). It has also been shown that nucleotides potentiate the induction of *c-Fos* expression by parathyroid hormone in the human osteoblast-like cell line SaOS-2 and in primary human bone derived cells (Bowler et al., 1999).

In this study, we confirm the findings reported thus far and show that BzATP activation of P2X7 receptors lead to a rapid and dramatic induction of *c-Fos* expression. Within 30

minutes of BzATP treatment, we observed a  $279 \pm 48$  fold increase in *c-Fos* expression as compared to the vehicle treated cells. In contrast to the previous 2 genes presented, dexamethasone does not significantly affect this stimulatory effect (Figure 8). This further corroborates with our hypothesis that BzATP stimulation of P2X7 leads to an up-regulation of osteogenic genes as *c-Fos* 1) promotes proliferation of osteoblasts and 2) as a transcription factor, it may trigger downstream signaling events which may eventually lead to up-regulation of various osteogenic genes. The finding that dexamethasone does not have an inhibitory effect on *c-Fos* expression may be attributed to the specificity of dexamethasone action. It is possible that dexamethasone only affects a specific set of genes directly involved in osteoblast differentiation.

Additionally, we examined other transcription factors as well as genes that encode various matrix proteins expressed during osteoblast differentiation. As mentioned in the introduction, a series of transcriptional events control the commitment of mesenchymal stem cells to a mature osteoblast. To summarize, RUNX2 is a key transcription factor controlling osteoblast differentiation. Osterix (OSX) acts downstream of RUNX2 and is specific for early and late stages of osteoblast differentiation (Koga et al., 2005; Nakashima et al., 2002; Nishio et al., 2006). The combined expression of RUNX2 and OSX initiates development of the committed preosteoblast which is characterized by expression of COL1 and BSP. Subsequent induction of activating transcription factor 4 (ATF-4) together with OSX and various Wnt/ $\beta$ -catenin signaling components leads to development of the mature osteoblast which express COL1, alkaline phosphatase (ALP) and osteocalcin (OCN) (Grol et al., 2009).

Type 1 collagen (COL1) is a major organic constituent of the extracellular matrix in bone. Its main function is to provide scaffolding for the nucleation of hydroxyapatite crystals during calcification. Non-collagenous proteins aid in the organization of the collagen matrix and regulate the formation and growth of hydroxyapatite crystals. Osteocalcin is the most abundant non-collagenous bone matrix protein (Wolf, 1996). It is a small carboxyglutamate protein expressed preferentially by osteoblasts and can bind calcium ions (Boivin et al., 1990). Alkaline phosphatase hydrolyzes ester bonds in organic phosphate compounds under alkaline conditions and plays an important role in

bone calcification (Anderson, 1989). *Mmp9* encodes for matrix metalloproteinase 9 which is an enzyme responsible for degrading extracellular matrix in physiological and pathological processes. It has been shown that RUNX2 mediates transactivation of the *Mmp9* promoter site in MC3T3-E1 cells (Pratap et al., 2005).

In this study, we show for the first time that neither BzATP nor dexamethasone affect the expression of *Colla1*, *Bglap* (encoding osteocalcin), *Alpl* and *Mmp9* up to 6 hours (Figure 9). This does not rule out the possibility of late induction of these genes following P2X7 activation since the time course was limited to 6 hours in the present study. Genes involved in matrix formation and maturation may be induced days following P2X7 activation. Indeed, a recent study by Kariya et al. show that tension-force induced P2X7 activation resulted in the up-regulation of RUNX2 and OSX in earlier time points (3 h and 6 h respectively), while COL1 and BSP peaked at day 3 and day 7 respectively (Kariya et al., 2015). Moreover, the expression of genes encoding downstream matrix proteins expressed by mature osteoblasts, OPN, OCN and ALP, peaked on day 14 of culture (Kariya et al., 2015). It would be informative to extend the time course and observe the changes in the expression of matrix genes upon P2X7 activation.

We also looked at *c-Myc*, another regulator gene that codes for a transcription factor. The protein encoded by the *c-Myc* gene is a multifunctional nuclear phosphoprotein that plays a role in cell cycle progression, growth, differentiation and apoptosis. Similar to *c-Fos*, *c-Myc* is also a proto-oncogene as its expression is often up-regulated in various forms of cancer. *c-Myc* expression is activated upon various signaling pathways such as Wnt and EGF (Ran et al., 1986; Zhang et al., 2012). It is also shown to be dependent on BMP induced signaling as it was shown to increase in BMP induced osteogenesis (Piek et al., 2010; Sakano et al., 1997). Here we showed that *c-Myc* expression was not affected by either BzATP or dexamethasone up to 6 hours. This could be due to the possibility that *c-Myc* expression may be dependent on a signaling pathway distinct from those activated upon P2X7 activation.

There are a few limitations of this study that should be noted.

- a) Firstly, we used a mouse osteoblast precursor cell line. As there are inherent species-specific differences between a mouse and human osteoblastic cell line, this may translate to differences in molecular interactions in bone remodeling as well.
- b) Secondly, BzATP may not specifically activate P2X7 as it is known to also stimulate other P2X receptors such as P2X1 with high affinity. Some of the effects seen in the study may be a result of a compounded effect of P2X receptors.
- c) Thirdly, it would have been helpful to have a control (time 0 hours) sample of cells treated overnight with dexamethasone. During the optimization process, we changed conditions from simultaneous treatment with dexamethasone and BzATP to overnight pretreatment of dexamethasone. However, due to an oversight, time 0 samples for dexamethasone treatment groups were not collected. Thus, it is possible that dexamethasone alone affects the expression of certain genes irrespective of BzATP action. In such cases, we do not have the time 0 control with which to compare changes in gene expression. However, this does not affect our conclusions regarding the actions of dexamethasone on responses to BzATP. In the future, a time 0 control for dexamethasone-treated cells should be included.
- d) Lastly, findings in the calvaria bone cells may not be extended to the jaw bones as bone characteristics may be site-specific (Judex et al., 2004). The differences between the different bone types may translate to differences in bone mechanotransduction. Viciilli et al studied the dentoalveolar morphology in WT and P2X7 KO mice and found no statistically significant differences between the two mouse types (Viciilli et al., 2009a). They conclude that P2X7 does not seem to have a major effect on alveolar bone or tooth morphology and that its effects may be site-specific to long bones. However, in a separate study published in the same year by the same group, the role of the P2X7 in orthodontic mechanical loading was investigated via P2X7 KO mouse model (Viciilli et al., 2009b). They show that hyalinization and root and bone resorption were different in the 2 types

of mice (increased in the KO mouse) and conclude that P2X7 plays a significant role in orthodontic mechanotransduction (Viecilli et al., 2009b). Nevertheless, there is a possibility that our findings may not be extrapolated to alveolar bones.

In conclusion, we show for the first time that dexamethasone inhibits osteogenic activities in bone cells in response to mechanical stimulus. As we hypothesized, the expression of anabolic genes such as *Ptgs2* and *Dmp1* are stimulated by BzATP activation of the P2X7 receptor. However, this is inhibited by dexamethasone confirming that dexamethasone may indeed have an inhibitory role in osteoblast differentiation and activation and eventual bone formation (Figure 10). In contrast to *Ptgs2* and *Dmp1*, the expression of *c-Fos* is stimulated with BzATP but not significantly affected by dexamethasone. This may indicate that dexamethasone specifically inhibits the expression of particular genes. We examined various other genes involved in osteoblast activation and bone formation and did not find a significant effect of BzATP stimulation with these genes up to 6 hours. An extended time course performed in primary osteoblast differentiation study is necessary to see if indeed these genes are regulated via P2X7 activation at later time points and whether dexamethasone has an effect.

It is also possible that while a transient activation of P2X7 is osteogenic, sustained stimulation could inhibit osteoblast function and activity (Agrawal and Gartland, 2015). It has been shown that, while short term application of BzATP induces reversible membrane blebbing without the activation of the key apoptotic mediator caspase-3 in murine osteoblastic cells (Li et al., 2005; Panupinthu et al., 2007), longer agonist stimulus can cause extensive membrane blebbing and ultimately lead to apoptosis (Gartland et al., 2001).



### Suggestions for Future Studies

- a) An important follow up to this study would be to repeat the study with P2X7 specific antagonists. This will strengthen the argument that the effects seen here were due to activation of P2X7 rather than other P2 receptors.
- b) A differentiation study done in a primary osteoblast culture looking at a longer timeline post ATP treatment should clarify whether the expression level of the genes involved in matrix mineralization is affected.
- c) Future work should also assess the amount of bone formation in primary rat or mouse osteoblasts with BzATP treatment in the presence or absence of dexamethasone. We hypothesize that an increased bone formation will be seen with BzATP treatment and that dexamethasone will reverse these effects.
- d) It would also be informative to measure the level of PGE<sub>2</sub> in the cell supernatant with an enzyme-linked immunosorbent assay (ELISA) to confirm the downstream effects of BzATP induced COX2 expression.
- e) Repeating this study with PGE<sub>2</sub> and LPA receptor antagonist will help further clarify the downstream signalling events following P2X7 activation and help dissect the autocrine/paracrine mechanism of osteoblast activation.
- f) It will be of interest to perform a dose dependent study with ATP as well as dexamethasone. It is possible that downstream signaling events following ATP-mediated P2X7 activation are dose and duration dependent (Grol et al., 2013). Dexamethasone action may also vary depending on the dosage and the duration of pre-treatment. We can vary the dose and the duration of dexamethasone pre-treatment and study the effect on dexamethasone-induced inhibition of osteogenic gene expression and bone formation.
- g) Eventually, it will be helpful to reproduce this study in an animal model such as mouse. We can treat P2X7 KO and wild type mice with or without

dexamethasone treatment and observe the differences in bone remodeling upon mechanical loading by studying the response in bone.

Last section of this thesis will briefly discuss the clinical relevance of the current findings in orthodontics and bone physiology.

#### *Relevance of the Study in Orthodontic Tooth Movement*

There are few studies directly addressing the biological mechanisms underlying mechanotransduction in orthodontics. In this study, we set out to elucidate molecular interactions during bone remodeling occurring in orthodontic tooth movement in order to better understand the relationship between genes and transcription factors implicated in bone remodeling. Expanding our basic science knowledge of the underlying mechanisms of OTM will help improve orthodontic mechanotherapy to be more biologic and efficient. Any interference responsible for inhibiting or delaying the sequence of events following mechanical stimulation leading to bone remodeling can have a negative effect on OTM. Here we show that dexamethasone may inhibit osteoblast maturation and bone formation via its inhibitory action on osteogenic gene expression. This finding may help explain why patients who have been on long-term corticosteroid therapy may react differently to orthodontic forces (Kalia et al., 2004). Initially, reduced bone formation may translate to an increased rate of tooth movement during active orthodontic treatment but decrease long-term stability during the retention phase. Hence, orthodontic patients on corticosteroid therapy may be more prone to post-treatment relapse and it may be necessary to implement a more stringent retention measures.

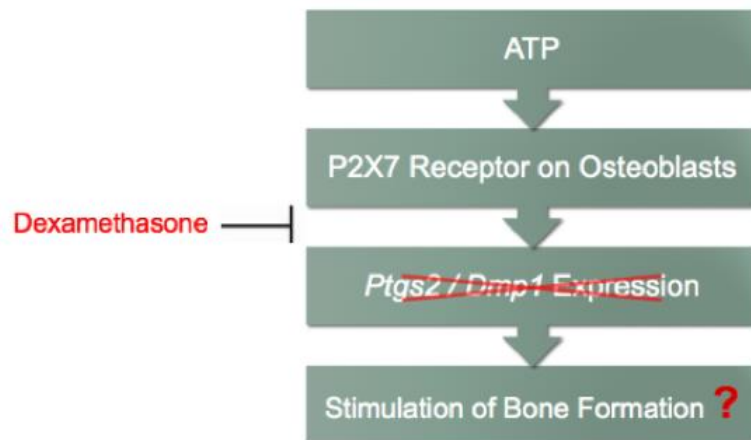
#### *Relevance of the Study in Glucocorticoid-Induced Osteoporosis*

Glucocorticoid-induced osteoporosis is a serious side effect of glucocorticoid therapy which can lead to fractures in 30 - 50% of patients (Fraser and Adachi, 2009). The mechanisms we have shown in this study may contribute to glucocorticoid-induced osteoporosis as we show that up-regulation of certain osteogenic genes on mechanical loading were inhibited by dexamethasone.

Furthermore, we suspect that patients suffering from glucocorticoid-induced osteoporosis may not respond favourably to exercise. Such patients may not react to mechanical loading the same way a normal healthy individual would. As observed in the study by Orriss et al, P2X7 activation in the presence of dexamethasone may trigger a distinct signaling pathway leading to inhibition of bone formation causing an imbalance in bone remodeling (Orriss et al., 2012; Orriss et al., 2013). Therefore, dexamethasone may possibly worsen the condition by inhibiting osteogenesis and promoting bone resorption.

## SUMMARY AND CONCLUSIONS

- We confirmed that the P2X7 agonist BzATP stimulates expression of *Ptgs2* in osteoblastic cells.
- We show, for the first time, that BzATP stimulates expression of *Dmp1*.
- We also show, for the first time, that BzATP stimulation of *Ptgs2* (Cox2) and *Dmp1* expression is inhibited by dexamethasone.
- BzATP dramatically stimulates *c-Fos* expression at early time points with no inhibition by dexamethasone. This indicates that the inhibitory effects of dexamethasone are specific for the expression of particular genes.
- In contrast, neither BzATP nor dexamethasone significantly affects the expression levels of *Colla1*, *c-Myc*, *Bglap*, *Alpl* or *Mmp9*.
- Consistent with our hypothesis, dexamethasone inhibits the ability of BzATP to stimulate the expression of two anabolic genes (*Ptgs2* and *Dmp1*) in osteoblastic cells).



**Figure 10. Summary of the findings**

We show for the first time that dexamethasone inhibits osteogenic activities in bone cells in response to mechanical stimulus. We confirmed that the P2X7 agonist BzATP stimulates expression of *Ptgs2* and *Dmp1* in osteoblastic cells. However, this is inhibited by dexamethasone confirming that dexamethasone may indeed have an inhibitory role in osteoblast differentiation and function, and eventual bone formation. In contrast to *Ptgs2* and *Dmp1*, the expression of *c-Fos* is stimulated with BzATP but not significantly affected by dexamethasone. This may indicate that dexamethasone specifically inhibits the expression of particular genes.

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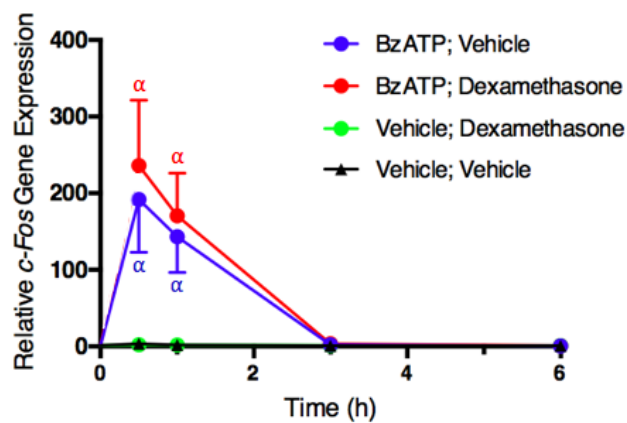
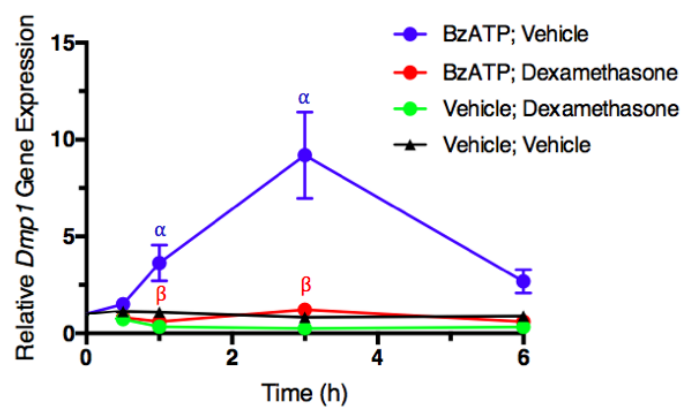
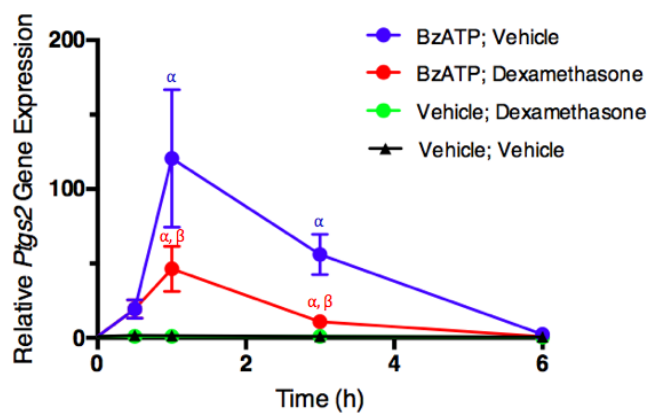
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## APPENDICES

**Appendix A: Results presented without the normalization as percentage of maximum gene expression.**

Data are shown as relative gene expression of respective genes.



## Appendix B: Permission to use Figure 4.

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